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Sent: Thursday, March 01, 2001 1:55 PM
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WO9618102A1

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308-4239
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09/368,010

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PCT

特許協力条約に基づいて公開された国際出願

(51) 国際特許分類6		(11) 国際公開番号	WO96/18102
G01N 33/53	A1	(43) 国際公開日	1996年6月13日(13.06.96)

(21) 国際出願番号　PCT/JP95/02427
(22) 国際出願日　1995年11月29日(29.11.95)

(30) 優先権データ
特願平6/303395　1994年12月7日(07.12.94)　JP

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AU, CA, CN, JP, KR, US, 欧州特許(AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

添付公開書類　国際調査報告書

(54) Title : METHOD OF MEASURING THE CONCENTRATION OF FK506-BINDING PROTEIN

(54) 発明の名称　FK506結合蛋白質濃度測定法

(57) Abstract

An enzyme immunoassay method for detecting FK506-binding proteins or measuring the concentration thereof by detecting or quantifying a complex comprising an FK506-binding protein and the first and second antibodies that recognize respectively different antigenic determinants of the protein.

(57) 要約

FK506結合蛋白質中のそれぞれ異なる抗原決定基を認識する第一抗体及び第二抗体と、FK506結合蛋白質とからなる複合体を検出しまたは定量することを特徴とするFK506結合蛋白質の検出、または濃度測定の為の酵素免疫測定法。

情報としての用途のみ

PCTに基づいて公開される国際出願をパンフレット第一頁にPCT加盟国を同定するために使用されるコード

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LT リトアニア
LU ルクセンブルグ
LV ラトヴィア
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MD モルドバ
MG マダガスカル
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MR モーリタニア
MW マラウイ
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TM トルクメニスタン
TR トルコ
TT トリニダード・トバゴ
UA ウクライナ
UG ウガンダ
US 米国
UZ ウズベキスタン共和国
VN ヴィェトナム

明 細 書

FK506結合蛋白質濃度測定法

「技術分野」

本発明は、FK506結合蛋白質の検出又はその濃度測定法、およびその検出又は濃度測定用キットに関するものであり、医療の分野で利用できる。

「背景技術」

FK506もしくはFR-900506とも表記される化合物は、強力な免疫抑制作用を有し、臓器移植時の拒絶反応や自己免疫疾患の予防剤または治療薬として使用しうることはよく知られている（例えば特開昭61－148181号）。

しかしながら、その作用は、非常に強力であるため、最適投与量の決定は重要な問題であり、副作用等を発生させることなく、効果的な免疫抑制活性を発揮させ得る量を投与することが極めて重要である。

一方、その後の研究により、FK506の免疫抑制作用はペプチジルプロリルシストランスイソメラーゼと同様な活性を有する、細胞内のFK506結合蛋白質(以下、FKBPと表記）と結合することにより発揮すると考えられている［例えば、Nature, 357, 692－694および695－697（1992）］。

該FKBPは、その分子量の違いによって数種のタイプが存在することが知られており、例えばFKBP－12（分子量12KDa）、FKBP－13（分子量13KDa）、FKBP－25（分子量

25KDa）、FKBP－52（分子量56KDa）等が報告され、それぞれの構造も既に決定されている。

たとえば、Nature, 341,755－757および758－760（1989）, J. Am. Chem. Soc. 113,1409－1411 (1991), Proc. Natl. Acad. Sci. 88,6229－6233（1991）等参照。

また、分子量11,819ダルトンで107個のアミノ酸からなるFKBPは遺伝子工学技術を用いて製造する手段も既に報告されている［Nature, 346,671－674（1990）］。

一方、それらFKBPの中でFK506が最も強く結合するのは、FKBP－12であり、その親和性定数（Kd）は0.4nMであること、更に、FKBP－12はリンパ球のみならず、赤血球を含めたあらゆる組織に多く存在していることも報告されている［Transpl. Proc. 23(6), 2760－2762（1991）］。

ところで、マウスMLRの系で、FKBP－12を一定量のFK506と共に添加すると、添加するFKBP－12の量に比例して、FK506の免疫抑制効果が阻害されることにより、血中にFKBP－12が存在すれば、FK506が血漿中のFKBP－12に結合され、その免疫抑制効果が血漿中濃度から予想される程、得られないことになる。手術後などでは、患者の赤血球が溶血し、赤血球のFKBP－12が血漿中に循環すること、及び、患者によりFK506に対する感受性に差があることを考えれば、患者のFK506感受性を推測し、より望ましいFK506血漿中濃度を設定する為に、血漿中

FKBP濃度を正確に測定する技術が望まれている。

血漿中FKBP濃度を測定する技術としては、国際公開公報94／04700に2種類のFKBP濃度の測定法が開示されている。1つには、FKBP中の抗原決定基を認識するが、FKBPとFK506との結合には影響を与えることのない抗FKBP抗体を固定化し、これに(1)被検試料中のFKBPを反応させ、次に(2)酵素で標識されたFK506を作用させ、更に(3)その酵素活性を測定することを特徴とするFKBP濃度の測定法、もう1つには、固定化したFKBPに、(1)被検試料および酵素で標識されたFK506を作用させることにより固定化したFKBPと、被検試料中のFKBPとの競合反応を行わせ、(2)固定化したFKBPに捉えられている酵素標識FK506の酵素活性を測定することを特徴とするFKBP濃度の測定法が開示されている。

しかし、これらの方法では、FKBPの有するFK506との結合能力を利用しFKBP濃度を定量する方法であるため、FK506が共存する被検試料中のFKBP濃度を正確に測定することは困難であった。

そこで、FK506が共存していても被検試料中のFKBP濃度を正確に測定できる技術の開発が望まれていた。

「発明の開示」

本発明の発明者等は、鋭意研究の結果、FKBPの抗原決定基を認識するモノクローナル抗体を利用し、血清や血漿のような被検試料中のFKBPの検出またはその濃度を正確に測定する方法、ならびに検出またはその濃度測定用

キットを確立した。

従って、本発明の目的は、被検試料中のFKBPを検出またはその濃度を正確に測定する酵素免疫測定法を提供すること、さらには、その検出、またはその濃度測定に供する測定用キットを提供することである。

すなわち、本発明は、

(1) 固相に固定化された第一抗体（FKBP中の抗原決定基を認識するモノクローナル抗体）、

(2) 被検試料中のFKBP、及び

(3) 第二抗体（FKBP中の抗原決定基を認識するが、上記第一抗体とは異なる抗原決定基を認識するモノクローナル抗体）

からなる複合体を形成させた後、その複合体を測定することからなるFKBPの検出、またはその濃度測定法、及び、前記第一抗体、第二抗体及び標準品としてのFKBPを含有するキットに関する。

FKBP中の抗原決定基を認識する第一抗体及び第二抗体は、例えば、ケーラーとミルスタイン（Kohler and Milstein）の基本方法［Nature, 256, 495 (1975)］のような常法の細胞融合法より製造することができる。

好ましくは、FKBPで免疫したマウスから得られた脾細胞と、マウス骨髄腫細胞とを細胞融合してハイブリドーマを製造し、その中から後述の製造例1あるいは製造例2のような方法で、FKBPを認識するモノクローナル抗体を調製することができる。より好ましくは、IgGやIgMの

クラスであり、最も好ましくは、$IgG_1\lambda$や$IgG_1\kappa$のようなサブクラスである。本願明細書記載の方法により、特に12KDaのFKBPのみと反応するか、または12Kd及び30～35Kdの両方のFKBPと反応する抗FKBPモノクローナル抗体が得られる。該方法は、国際公開公報94／04700及びTranspl. Proc. 25,655－657（1993）により既に公知である。

尚、上記のようにして得られるモノクローナル抗体のうち、FKBP中のそれぞれ別の抗原決定基を認識する2つの型のモノクローナル抗体を選択し、第一抗体及び第二抗体として各々使用することが出来る。

そして、特に好ましい第一抗体及び第二抗体用のモノクローナル抗体としては、FKBPがFK506と結合していても何ら影響を受けることなく、FKBP中のそれぞれ別の抗原決定基を認識するモノクローナル抗体が挙げられる。

なお、本発明における「第二抗体」なる用語は、前記のように第一抗体とは異なるＦＫＢＰ中の抗原決定基を認識するモノクローナル抗体そのものに加え、複合体の検出、または定量時において使用される適当な標識を有する前記モノクローナル抗体をも意味する。

本発明における「標識を有する第二抗体」における標識としては、そのものの存在が検出されうるものであればよいが、たとえば酵素、ある蛋白質に親和性を有する化合物（例えばビオチン）、第三抗体（第二抗体を認識する抗体）、放射性同位元素、螢光物質、発光物質等が

挙げられる。

酵素としては、安定で比活性の大きなものが好ましく、その例としてはたとえば(1)カルボヒドラーゼ[例、グリコシダーゼ(例、β－ガラクトシダーゼ、β－グルコシダーゼ、β－グルクロニダーゼ、β－フルクトシダーゼ、α－ガラクトシダーゼ、α－グルコシダーゼ、α－マンノシダーゼ)、アミラーゼ(例、α－アミラーゼ、β－アミラーゼ、イソアミラーゼ、グルコアミラーゼ、タカアミラーゼA)、セルラーゼ、リゾチーム等]、(2)アミダーゼ(例、ウレアーゼ、アスパラギナーゼ)、(3)エステラーゼ[例、コリンエステラーゼ(例、アセチルコリンエステラーゼ)、ホスファターゼ(例、アルカリホスファターゼ)、スルファターゼ、リパーゼ等]、(4)ヌクレアーゼ(例、デオキシリボヌクレアーゼ、リボヌクレアーゼ)、(5)鉄・ポルフィリン酵素(例、カタラーゼ、ペルオキシダーゼ、チトクロームオキシダーゼ)、(6)銅酵素(例、チロシナーゼ、アスコルビン酸オキシダーゼ)、(7)脱水素酵素(例、アルコール脱水素酵素、リンゴ酸脱水素酵素、乳酸脱水素酵素、イソクエン酸脱水素酵素)などが挙げられ、とりわけ、アルカリフォスファターゼが好ましい。

ビオチンは、ビタミンHとして知られる化合物であり、卵白中に存在する塩基性蛋白質であるアビジンと極めて高い親和性を有している。アビジンは、4個のサブユニットから成ることが知られているが、本発明のアビ

ジンとはこれらのサブユニットおよび前記酵素（好ましくは、アルカリフォスファターゼ）で標識されたアビジンをも含む。

第二抗体を認識する抗体である第三抗体としては、たとえば、抗マウスラムダ鎖抗体、抗マウスカッパー鎖抗体などの抗免疫グロブリン抗体などが挙げられ、そのうち前記標識を有していてもよい、とりわけ酵素（アルカリフォスファターゼ）を有する抗マウスラムダ鎖抗体が好ましい。

放射性同位元素としてはたとえば^{125}I，^{131}I，^{3}H，^{14}Cなどが挙げられる。

螢光物質としては、フルオレスカミン、フルオレッセンスイソチオシアネートなどが、発光物質としてはルミノール、ルミノール誘導体、ルシフェリン、ルシゲニンなどがそれぞれ挙げられる。

標識を有する第二抗体または第三抗体の調製に際しては、前記抗体と標識とを結合させうる公知の方法を用いることができるが、たとえばクロラミンT法、過ヨウ素酸法、マレイミド法などが用いられる。より具体的には、後記実施例の方法を用いることができる。

本発明におけるFK506結合蛋白質（FKBP）とは、前記のようなFKBP－12、FKBP－13、FKBP－25、FKBP－52等を意味する。

しかし、FK506が最も強く結合するのがFKBP－12であること、そしてFKBP－12があらゆる組織に多量に存在し

ていることを考慮すると、特に好ましいのはFKBP－12である。そして、その場合の第一抗体及び第二抗体とは、FKBP－12がFK506と結合していても何ら影響を受けることがなく、FKBP－12中のそれぞれ別の抗原決定基を認識するモノクローナル抗体である。

本発明において、第一抗体、FKBP及び第二抗体からなる複合体を定量するに際しては、慣用の種々の方法が可能であるが、特に酵素基質反応を用いる場合、例えば、次のような方法が考えられる。

（A）あらかじめ適当な酵素を用いて第二抗体を標識しておき、前記複合体を形成させた後、その酵素に応じた適当な酵素基質反応を行なう。

（B）第一抗体、FKBP及びビオチンを常法により結合させたビオチンで標識した第二抗体との複合体を形成させた後に、適当な酵素で標識したアビジンを反応させ、その標識酵素に応じた適当な酵素基質反応を行なう。

（C）あるいは、あらかじめ第二抗体を認識する酵素標識抗体（たとえば、アルカリフォスファターゼ標識抗マウスラムダ鎖抗体）と第二抗体との結合体を用意しておき、第一抗体、ＦＫＢＰ及びその結合体との複合体を形成させた後、適当な酵素基質反応を行なう。

酵素基質反応に使用される基質としては、酵素の種類によって選択することが出来る。例えば、４－メチルウ

ンベリフェリルフォスフェート、クロマーゲン、O－フェニレンジアミンなどを用いることが出来るが、好ましくは、4－メチルウンベリフェリルフォスフェートである。

そして、生成した複合体量を反映した酵素基質反応によって生じる基質の変化量を吸光度や螢光強度として慣用の方法により測定する。

また、被検試料がヒト血清、血漿及び組織抽出液の場合には、エチレンジアミン四酢酸二ナトリウム（EDTA・2Na）、クエン酸二ナトリウムのようなキレート試薬を添加することにより、より測定精度を上げることも可能である。

なお、第一抗体を固相に固定化する方法は第一抗体を固相とともに適当な時間放置するというような慣用の方法により行なわれ、その際の固相としては、モノクローナル抗体のような抗体を固定化出来、その後の反応液との分離作業を容易に出来るものであれば、慣用のものが使用できるが、好ましくはイムノプレートを用いることができる。

本願発明における第一抗体と被検試料中のFKBPとの反応体、あるいは更に第二抗体との複合体形成の際の反応は、通常の抗原抗体反応に適した条件において行われればよく、好ましくは、室温下数時間振とうすればよい。

より具体的なFKBPの検出、または濃度測定の手順は、後記の実施例と同様にして行なわれるが複合体を形成さ

せる際の、第一抗体、FKBPおよび第二抗体の添加順序は、後記の実施例の順序に限定されるものではない。

本発明により、種々の臓器や血中の被検試料中のFKBPを簡便に、より正確に検出または濃度を測定することが出来るようになったことにより、例えば、FK506の最適投与量を決定する際の重要な判断材料を提供することが可能となった。

そして、必要に応じ、ある特定のFKBP（例えば、FKBP－12）のみを選択的に検出したりまたは、その濃度を測定したり、全てのFKBPを検出しまたはその総濃度を測定出来るようになったため、実際の医療の場で、個々の患者の状態に応じたFK506の投与量をきめこまかく設定できるようになった。

特に、FKBPがFK506と結合していても、何ら影響を受けることなくFKBP中の抗原決定基を認識し得るモノクローナル抗体（例えば、後記実施例で使用したような抗FKBP－12モノクローナル抗体2C1－87，抗FKBP－12モノクローナル抗体3F4－70）を第一抗体及び第二抗体として、それぞれ用いる場合には、FK506が既に投与された患者の血清もしくは血漿から、FK506を前もって除去しなくてもFKBPを正確に簡便に検出またはその濃度を測定することが可能であるため、特に有用である。

以下、本発明を製造例、実施例により詳細に説明するが、本発明はこれらに限定されるものではない。

製造例1　抗FKBP抗体の製造

(1) FKBP－12の製造と特徴

Nature, 346, 671－674 (1990) の中でHarvard大学S. L. Schreiberらの報告しているDNA sequenceより、FKBP－12のC－末に相当するDNA 48merをApplied Biosystem社のDNA synthesizerを使って合成した。

5'－CCACATGCCACTCTCGTCTTCGATGTGGAGCTTCTAAAACTGGAATGA－3'

この48－merの末端を^{25}Pでラベルし、プローブとしてCLONTECH社のヒトT－cell cDNAライブラリーHL1016b 50万プラークをスクリーニングしたところ、ポジティブプラークを一つ得た。このプラークよりFKBP－12cDNAを含む断片をサブクローニングした［pUC－23 (Ec)］。このpUC－23 (Ec) をシークエンスしたところ、N－末DNAシークエンス1番から32番に相当する部分が欠損していたので、この部分を補完すると共に、大腸菌での発現を高くする為に合成したAT rich silent mutant N－末DNA約80b.p.とを利用し、EcoRI－BamHI siteとして、tryptophan promotorの制御下で発現するプラスミドに組み込み、発現ベクターpFKBP333を得た。これを、E. coli HB101に形質転換し、発現菌HB101／pFKBP (AT) 311を得た。これを、L－amp. brothにて19時間培養し、蛋白合成の誘発は、90μg／ml (final濃度) となる様にIAA (Indol－Acrylic acid) を添加することによ

り行った。E. coliを集菌し、50mM Tris－HCl, 2 mM β－ME, 2 mM $CaCl_2$, 10mM $MgCl_2$, 5 % glycerol中でFrench Pressにて細胞を粉砕し、遠心（4 ℃, 6,000×g, 30分）－上清を60℃, 15分間熱処理－遠心（4 ℃, 6,000×g, 45分＋4 ℃, 18,000×g, 20分×2回）－透析［20mM Tris－HCl（pH7.4），4 ℃　終夜］－DEAE－Toyo PEARL 650M－逆相HPLC（C 4）にてFKBP－12を精製した。

(2) FKBP－12のマウスへの免疫

FKBP－12のリン酸緩衝液（以下、PBSと表記）溶液250μg／ml 0.1mlと等量の Freund's Complete Adjuvant（FCA）を混合し、マウスBALB／cの腹腔内に免疫した。同量のFKBP－12をFreund's Incomplete Adjuvant（FIA）と混合し、およそ10日毎に数回、腹腔内に免疫した。

(3) 血中抗体価の測定

マウス尾静脈より10μlを採血し、PBS 990μlと混合し、遠心後測定試料とした。測定に当たっては、10μg／mlのFKBP－12 PBS溶液50μlをイムノプレートウェルに加え、室温3時間反応させ、表面に結合させる。その後、ウェルを洗浄、0.2%ミルクブロッカーPBSで非特異的結合サイトをブロックし、再び洗浄後、試料希釈液50μlを加え、室温下1時間反応させる。洗浄後、anti－mouse IgG（H＋L）－POD［ベクター（Vector Lab.）社製］100μl／ウェル添加し、室温で更に1時間反応さ

せる。洗浄後、常法によりO－フェニレンジアミンの系で発色させた。

(4) 抗FKBP－12抗体の産生

抗体価の上昇が見られたマウスに更に最終免疫としてFKBP－12 250μg／ml (PBS) 0.2mlを尾静脈から注射した。4日後に脾臓を抽出し、脾臓細胞1.44×10^8cellsを調整した。一方、マウス骨髄腫細胞P3X63Ag8U.1を2.9×10^7cell調整し、50％PEG4000中（最終濃度）で細胞融合を行った。その後、HAT培地にて24ウェルプレート上、10^6 cells／ml×1ml／ウェルで融合細胞のスクリーニングを行った。2週間後、HAT培地で細胞の成育が確認できた144ウェルについて、抗FKBP－12抗体の産生を血中抗体価の測定法によってスクリーニングした。つまり、血中抗体価の測定法において、抗FKBP－12抗体を含む試料を添加反応させる時に、final 5μg／mlのFKBP－2を共存させ、FKBP－12存在下での発色が消失するものを選択した。更にFKBP－12に対して抗体価の高いクローン32クローンを選択した。

(5) FKBP－12を認識し、FK506, FK506－C32 (LA)－PODとFKBP－12との結合を阻害しない抗FKBP－12抗体の選択

固相に抗マウスIgG (H＋L) を結合させ、更にスクリーニングの抗体を結合させた後、1μg／mlのFKBP－12 50μlと下記(6)で得られたFK506－C33 (LA)－POD（1000倍希釈）50μlとを共存させた。O－フェニレン

ジアミンとの反応により生じた490nmでの吸光度の上昇が見られたクローンを選択した。この時、10μg/mlのFK506を共存させた時の発色の消失により、抗マウスIgG(H+L)により結合されたFKBP－12はFK506－C32(LA)－PODのFK506を認識していることが分かる。その結果、FKBP－12をそのまま抗原として用いた免疫法からは5種類の抗体(5－1－A5, 5－1－B3, 5－1－C5, 5－2－D1 (IgG_1), 5－4－D2)、下記(7)のようにFKBP－12を尿素で変性させたものを抗原として用いた免疫法からはIA4 (IgM), 3A8 (IgG_2)、IF7、3B8、また、下記(8)のようにFKBP－12をオブアルブミンと結合させたものを抗原として用いた免疫法からは4F8の合計10種のモノクローナル抗体が得られた。

(6) FKBP506－C32(LA)－PODの製造法

特開平1－92659号の実施例1と同様にて得られたコハク酸－FR－900506物質ハーフエステル(230mg)、N－ヒドロキシサクシンイミド(35mg)及び1－エチル－3－(3－ジメチルアミノプロピル)カルボジイミド塩酸塩(43mg)の塩化メチレン溶液(10ml)を室温下5時間攪拌した後、反応混合物を水洗し、さらに乾燥した。溶媒を留去した後、得られた残渣(250mg)を11－アミノウンデカン酸(120mg)及びトリエチルアミン(60mg)とともにジメチルホルムアミド－水(1:1, 20ml)溶媒中で室温下6時間攪拌した。反応混合物は水洗

後乾燥した上で溶媒を留去し、得られた残渣をクロロホルムを展開溶媒とするシリカゲルカラムに付し、17−アリル−1,14−ジヒドロキシ−12−［2−（4−N−（10−カルボキシデシル）−（4−アミノ−1,4−ジオキソ−1−ブチルオキシ））−3−メトキシシクロヘキシル）−1−メチルビニル］−23,25−ジメトキシ−13,19,21,27−テトラメチル−11,28−ジオキサ−4−アザトリシクロ［22.3.1.$0^{4,9}$］オクタコス−18−エン−2,3,10,16−テトラオン（120mg）を得た。

NMR（$CDCl_3$，δ）：1.2-1.4（m），5.9-6.1（1H,m）

・FAB MASS：1109（M+Na)$^+$

更に、上記化合物を用い、特開平1−92659号の実施例1の3）と同様にしてホースラディッシュ・ペルオキシダーゼを反応させることにより、FK506−C32（LA）−POD溶液を得た。

（7）尿素変性FKBP−12の調製

FKBP−12の0.08%トリフルオロ酢酸含有水溶液（847μg／ml）460μlに尿素312mgを添加し、室温下、混合撹拌し、FKBP−12（600μg／ml）の溶液650μlを調製した。これを、このまま免疫に使用した。

（8）オブアルブミン結合FKBP−12の調製

FKBP−12 1032μg（1mg／mlを1032μl使用）とオブアルブミン（シグマ社、Lot No.76F−8040）のPBS溶液（2mg／ml）1032μlを混合する。これに、0.13Mグルタルアルデヒド−PBS溶液0.62mlを滴下する。室温下、

14時間攪拌し、その後、PBS1lに対し、3回透析し、これを免疫原として使用した。

FKBP－12とオブアルブミンとの混合比率は、FKBP－12 1032μg：オブアルブミン2064μgで、溶液量は2.7mlであった。

製造例2　抗FKBP－12抗体の製造

(1) 製造例1の(1)および(2)のようにしてマウスに免疫した。

(2) 血中抗体価の測定

20μg／mlのFKBP－12のPBS溶液50μlをELISA用96ウェルプレートの各ウェルに分注し、4℃で一晩放置した。ウェルのFKBP－12溶液を吸引除去し、0.05％Tween20／PBS溶液で3回洗浄した。プレートの各ウェルに0.2％ミルク／PBS溶液250μlを加え、室温で30分間放置した。ウェルの0.2％ミルク／PBS溶液を吸引除去し、0.2％ミルク／0.05％Tween20／PBS溶液で希釈した各抗血清を100μl、ウェルに加え、室温で2時間放置した。次いで、ウェルの抗血清希釈液を吸引除去し、0.05％Tween20／PBS溶液で3回洗浄した。0.2％ミルク/0.05％Tween20／PBS溶液で1000倍に希釈したアルカリフォスファターゼ標識抗マウスIgG（H＋L）溶液を各ウェルに100μl加え、室温で2時間放置した。ウェルのアルカリフォスファターゼ標識抗マウスIgG（H＋L）溶液を吸引除去し、0.05％Tween20／PBS溶液で5回洗浄した。その後、0.1mM 4－メチルウンベリフェリルフォスフェー

ト（4MU－P；シグマ社）の緩衝液（10mMジエタノールアミン／0.5％$MgCl_2$／H_2O）溶液を各ウェルに100μl加え、室温で15分間放置した。各ウェルの螢光強度（Exitation；360nm, Emission；460nm）を螢光プレート・リーダーで測定した。

(3) 抗体価の一番強いマウスを選択し、4回目注射の10日後に最終免疫として1.5mg／mlのFKBP－12のPBS溶液0.2mlをマウスの尾静脈から注射した。

(4) 細胞融合

製造例1－(4)と同じ方法で細胞融合を行い、ハイブリドーマ（3－3－D4－C6，2C1－87及び3F4－70）を得た。

(5) 抗体の産生と精製

スクリーニング及びクローニングで得られたハイブリドーマ2C1－87、あるいはハイブリドーマ3F4－70を、それぞれF75フラスコに5×10^4個／ml（50ml／F75）になるように播種し、4日間培養後、培養液を遠心し、血清無添加培地で2回洗浄後、それぞれ0.5mlに浮遊させた。2週間前に、プリスタン0.5mlを腹腔内に注射したBALB／Cマウス（BALB／C，雌，6週令）の腹腔内へハイブリドーマ浮遊液0.5mlを移植した。10日後、マウスを開腹し、それぞれのマウスから腹水を5ml得た。それぞれの腹水をアフィゲルプロテインA MAPS－IIキット（バイオラド）にて精製を行い、抗FKBP－12モノクローナル抗体2C1－87（サブタイプ：IgG_1

λ）及び3F4－70（サブタイプ：IgG$_1$κ）精製抗体を得た。また、ハイブリドーマ3－3－D4－C6を同様に培養後、抗FKBP－12モノクローナル抗体3－3－D4－C6（サブタイプ：IgM）を得た。

実施例1．FKBP－12の酵素免疫測定法（AP－McAb法）

1）モノクローナル抗体のアルカリフォスファターゼ標識

精製された1mgの抗FKBP－12モノクローナル抗体2C1－87あるいは3F4－70をイムノリンクーアルカリフォスファターゼーラベリング キット（ケンブリッジ バイオケミカル社製，コードCK－07－1000）添付記載の方法に従ってアルカリフォスファターゼ標識を行い、各標識抗体1mlを得る。

2）抗体の固相への吸着操作

モノクローナル抗体2C1－87もしくは3F4－70のPBS溶液（5μg／ml）50μlずつをエリザ用プレート（マキシソープF96，ヌンク社製）の各ウェルに分注し、4℃で一晩静置する。プレートを0.05％ツイーン20のPBS溶液で3回洗浄する。

3）特異的吸着防止操作

上記の各プレートに，4％ブロックエース（雪印社製，コードUK－B80）の水溶液を250μl分注し，室温で30分放置後、プレートを0.05％ツイーン20のPBS溶液で1回洗浄する。

4）抗原抗体反応

4％ブロックエース－0.1％ツイーン20の水溶液で希釈した標準FKBP－12溶液100μlずつを上記プレートの各ウェルに分注し、プレートミキサーで振とうしながら室温で2時間置く。プレートを0.05％ツイーン20のPBS溶液で7回洗浄する。

5）標識抗体反応

1％ブロックエース－0.1％ツイーン20の水溶液で1000倍に希釈したアルカリフォスファターゼ標識モノクローナル抗体を100μlずつ上記プレートの各ウェルに分注し，プレートミキサーで振とうしながら室温で2時間置く（2C1－87固相の場合は3F4－70標識抗体，3F4－70固相の場合は2C1－87標識抗体）。プレートを0.05％ツイーン20のPBS溶液で7回洗浄する。

6）酵素反応

下記のように調製した酵素基質溶液（100μl）を上記プレート各ウェルに分注し，30分間室温で反応させる。酵素基質溶液は使用直前に調製する。

酵素基質溶液：

0.5mM 4－メチルウンベリフェリルフォスフェート

ジエタノールアミン	10 ml
塩化マグネシウム・1水和物 $(MgCl_2 \cdot H_2O)$	10 mg
蒸留水	90 ml

7）測定

標準FKBP－12溶液の代わりに4％ブロックエース－0.1％ツイーン20の水溶液を加えたウェルを対照とし、サイトフロー（商品名，ミリポア社製）により蛍光強度（Ex. 360nm, Em. 460nm）を測定する。

以上の測定法の結果を表1に示す。

表1　FKBP－12の酵素免疫測定法（AP－McAb法）結果

FKBP－12濃度（ng／ml）	第一抗体（蛍光強度）	
	3F4－70	2C1－87
500	5168	4516
250	4434	3612
125	2979	2083
62.5	1837	1158
31.25	909	432
15.625	402	162
7.8125	155	48

実施例2．FKBP－12の酵素免疫測定法（ビオチン－McAb法）

1）モノクローナル抗体のビオチン標識

精製2mgの抗FKBP－12モノクローナル抗体2C1－87あるいは3F4－70を7.5％重炭酸ナトリウム緩衝液（pH8.5）溶液1ml に溶解し10mg／mlのBNHS（N－hydroxysuccinimidyl－6－（biotinamido）hexanoate，ベクター社製，コードSP－1200）のDMSO溶液20μlを加え，時々撹拌しながら，室温で2時間反応させた後，PBSに対して一晩透析し，ビオチン標識抗体1mlを得る。

2）抗体の固相への吸着操作

モノクローナル抗体2C1－87もしくは3F4－70のPBS溶液（5μg／ml）50μlずつをエリザ用プレート（マキシソープF96，ヌンク社製）の各ウェルに分注し，4℃で一晩静置する。プレートを0.05％ツイーン20のPBS溶液で3回洗浄する。

3）特異的吸着防止操作

上記の各プレートに，4％ブロックエース（前記述）の水溶液を250μl分注し，室温で30分放置後，プレートを0.05％ツイーン20のPBS溶液で1回洗浄する。

4）抗原抗体反応

4％ブロックエース－0.1％ツイーン20の水溶液で希釈した標準FKBP－12溶液100μlずつを上記プレートの各ウェルに分注し，プレートミキサーで振とうしながら室温で2時間置く。プレートを0.05％ツイーン20のPBS溶液で7回洗浄する。

5）標識抗体反応

1%ブロックエース－0.1%ツイーン20の水溶液で1000倍に希釈した上記ビオチン標識モノクローナル抗体を100μlずつ上記プレートの各ウェルに分注し，プレートミキサーで振とうしながら室温で2時間置く（2C1－87固相の場合は3F4－70標識抗体，3F4－70固相の場合は2C1－87標識抗体）。プレートを0.05%ツイーン20のPBS溶液で7回洗浄する。

6）ビオチン－アビジン反応

1%ブロックエース－0.1%ツイーン20の水溶液で1000倍に希釈したアルカリフォスファターゼ標識アビジン溶液（ベクター社製，コードA－2100）を上記プレートの各ウェルに分注し，プレートミキサーで振とうしながら室温で30分間置く。プレートを0.05%ツイーン20のPBS溶液で7回洗浄する。

7）酵素反応

下記のように調製した酵素基質溶液（100μl）を上記プレート各ウェルに分注し，30分間室温で反応させる。酵素基質溶液は使用直前に調製する。

酵素基質溶液：

0.5mM 4－メチルウンベリフェリルフォスフェート

ジエタノールアミン	10 ml
塩化マグネシウム・1水和物 ($MgCl_2 \cdot H_2O$)	10 mg
蒸留水	90 ml

8）測定

標準FKBP－12溶液の代わりに4％ブロックエース－0.1％ツイーン20の水溶液を加えたウェルを対照とし、サイトフロー（商品名，ミリポア社製）により螢光強度（Ex. 360nm, Em. 460nm）を測定する。

以上の測定法の結果を表2に示す。

表2　ＦＫＢＰ－１２の酵素免疫測定法（ビオチン－ＭｃＡｂ法）結果

ＦＫＢＰ－１２濃度（ng／ml）	第一抗体（螢光強度）	
	３Ｆ４－７０	２Ｃ１－８７
1000	２３９１	７１３
500	１９１３	４１７
250	１２８８	２４１
125	７３６	１２５
62.5	３８２	６０
31.25	１７５	２４
15.625	７８	――
7.8125	１４	――

実施例3．FKBP－12の酵素免疫測定法（AP－antiλ－McAb法）1）抗体の固相への吸着操作

モノクローナル抗体3F4－70のPBS溶液（5μg／ml)50μlずつをエリザ用プレート(マキシソープF96，ヌンク社製）の各ウェルに分注し，4℃で一晩静置する。プレートを0.05％ツイーン20のPBS溶液で3回洗浄すでる。

2）特異的吸着防止操作

上記の各プレートに，4％ブロックエース(前記述）の水溶液を250μl分注し，室温で30分放置後，プレートを0.05％ツイーン20のPBS溶液で1回洗浄する。

3）抗原抗体反応

4％ブロックエース－0.1％ツイーン20の水溶液で希釈した標準FKBP－12溶液100μlずつを上記プレートの各ウェルに分注し，プレートミキサーで振とうしながら室温で2時間置く。

4）2C1－87，AP－antiλ結合体作製

モノクローナル抗体2C1－87を5μg／mlになる様に1％ブロックエース－0.1％ツイーン20水溶液で希釈しこれに5000倍希釈になるようにアルカリフォスファターゼ標識抗マウスラムダ鎖抗体溶液（サザンバイオテクノロジー社製，コード1060－04）を加え2時間放置する。

5）結合抗体反応

プレートを0.05%ツイーン20のPBS溶液で7回洗浄し，4）で作製した結合抗体を100μlずつ上記プレートの各ウェルに分注し，プレートミキサーで振とうしながら室温で2時間置く。プレートを0.05%ツイーン20のPBS溶液で7回洗浄する。

6）酵素反応

下記のように調製した酵素基質溶液（100μl）を上記プレート各ウェルに分注し，30分間室温で反応させる。酵素基質溶液は使用直前に調製する。

酵素基質溶液：

0.5mM 4－メチルウンベリフェリルフォスフェート

ジエタノールアミン	10 ml
塩化マグネシウム・1水和物	
($MgCl_2 \cdot H_2O$)	10 mg
蒸　留　水	90 ml

7）測定

標準FKBP－12溶液の代わりに4%ブロックエース－0.1%ツイーン20の水溶液を加えたウェルを対照とし、サイトフロー（商品名，ミリポア社製）により蛍光強度（Ex. 360nm, Em. 460nm）を測定する。

以上の測定法の結果を表3に示す。

表３　ＦＫＢＰ－１２の酵素免疫測定法
（ＡＰ－ａｎｔｉλ－ＭｃＡｂ法）

ＦＫＢＰ－１２ 濃 度 （ng／ml）	蛍 光 強 度
500	３３１６
250	２２６３
125	１３１４
62.5	７０７
31.25	３０９
15.625	１４７
7.8125	８６

実施例４．ヒト血清中のFKBP－12濃度の測定

実施例２の４）において特異的吸着防止操作を終了したプレートの各ウェルに50μlの0.4％エチレンジアミン四酢酸二ナトリウム，２水和物（EDTA－２Na），４％ブロックエース，0.1％ツイーン20を含む水溶液（以後アッセイバッファーと記述）を加えたのち，４％ブロックエース，0.1％ツイーン20の水溶液（以後アッセイ希釈液と記述）で希釈したFKBP－12を標準溶液とし，ヒト血清（対照）あるいはFKBP－12を25ng／mlになるよう

に添加したヒト血清を50μl加える。血清中の添加FKBP−12の濃度を標準曲線より計算した。結果を表4に示す。

表4．ヒト血清中のＦＫＢＰ−１２濃度測定

	ＦＫＢＰ−１２濃度測定値 (ng／ml)
1	２９．４５
2	２３．３２
3	３２．９３
4	３３．７７

実施例５．採血法の異なる検体の血中FKBP−12濃度の測定

病態の異なる患者（Ａ，Ｂ，Ｃ）から異なる採血法で血液を採取し血中FKBP−12濃度を測定した。実施例３の4)において特異的吸着防止操作を終了したプレートの各ウェルに50μlのアッセイバッファーを加えたのち，アッセイ希釈液で希釈した標準FKBP−12あるいは同一患者から採取した血清，ヘパリン採血血漿，EDTA−Na採血血漿をアッセイ希釈液で３倍に希釈した検体50μlをそれぞれウェルに加え実施例３にしたがって測定した。患者血清中のFKBP−12の濃度を標準曲線より計算した。結果を表５に示す。

表５．採血法の異なる血中ＦＫＢＰ－１２濃度の測定

	採血方法／ＦＫＢＰ－１２濃度（ng／ml）		
患者	ＥＤＴＡ－Ｎａ採血血漿	ヘパリン採血血漿	血清
A	５８．６９	６２．３８	５９．４８
B	５４７．５	５００．５	５３８．９
C	５３．６５	５２．５０	５４．４３

実施例６．肝臓移植患者のFKBP－12濃度の測定

ある肝臓移植患者からヘパリン採血し血漿を得て、実施例５と同様に血中FKBP－12濃度を測定した。結果を表６に示す。

表６．肝臓移植患者の血中ＦＫＢＰ－１２濃度の測定

手術後の日数	血中ＦＫＢＰ－１２濃度（ｎｇ／ｍｌ）
手術前	＞３０００
４日	４５９．１
５日	２３４．５
７日	２５０．０
１１日	４４３．５
１４日	１５３．３
１７日	２７０．４
１８日	８１．１１

請求の範囲

1．固相に固定化された第一抗体（FK506結合蛋白質中の抗原決定基を認識するモノクローナル抗体）、被検試料中のFK506結合蛋白質および第二抗体（FK506結合蛋白質を第一抗体とは異なった抗原決定基で認識するモノクローナル抗体）からなる複合体を形成させた後、その複合体の存在または量を検出または測定することを特徴とするFK506結合蛋白質の検出または濃度測定方法。

2．第一抗体および第二抗体であるモノクローナル抗体がFK506結合蛋白質とFK506との結合を阻害することのないモノクローナル抗体である請求の範囲第1項記載の方法。

3．FK506結合蛋白質がFKBP－12である請求の範囲第1項または第2項記載の方法。

4．第二抗体が標識を有する第二抗体であり、その標識を利用することにより、複合体の存在または量を検出または測定することを特徴とする請求の範囲第1項、第2項または第3項記載の方法。

5．標識が酵素であり、その酵素基質反応を利用する請求の範囲第4項記載の方法。

6．標識がビオチンであり、そのビオチンと反応するアビジンを作用させ、結合したアビジンの存在または量を検出または測定することを特徴とする請求の範囲第4項記載の方法。

7．結合したアビジンの存在または量を検出または測定

するに際し、アビジンを標識する酵素を利用する請求の範囲第6項記載の方法。

8. 標識が第三抗体（第二抗体を認識する抗体）である請求の範囲第4項記載の方法。

9. 第三抗体が抗マウスラムダ鎖抗体である請求の範囲第8項記載の方法。

10. 抗マウスラムダ鎖抗体が標識を有する抗マウスラムダ鎖抗体である請求の範囲第9項記載の方法。

11. 標識を有する抗マウスラムダ鎖抗体が酵素で標識された抗マウスラムダ鎖抗体である請求の範囲第10項記載の方法。

12. 酵素がアルカリフォスファターゼである請求の範囲第5項、第7項または第11項記載の方法。

13. 被検試料がキレート試薬を添加した被検試料である請求の範囲第4項記載の方法。

14. 固相に固定化された第一抗体（FK506結合蛋白質の抗原決定基を認識するモノクローナル抗体）に、(1) FK506結合蛋白質を含有するまたは含有する疑いのある被検試料を作用させ、ついで(2)第二抗体（第一抗体とは異なるFK506結合蛋白質の抗原決定基を認識するモノクローナル抗体）を作用させ、その後(3)生成した複合体の存在または量を検出または測定することを特徴とする請求の範囲第1項記載の方法。

15. FK506結合蛋白質、第一抗体および第二抗体を含有するキット。

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP95/02427

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl^6 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl^6 G01N33/53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Jitsuyo Shinan Koho 1926 - 1995
Kokai Jitsuyo Shinan Koho 1971 - 1995

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JP, 64-59068, A (Tosoh Corp.), March 6, 1989 (06. 03. 89) (Family: none)	1 - 15
Y	WO, 94/04700, A1 (Fujisawa Pharmaceutical Co., Ltd.), March 3, 1994 (03. 03. 94) & EP, 672756, A1	1 - 15
A	JP, 63-49095, A (Unitika Ltd.), March 1, 1988 (01. 03. 88) & EP, 261781, A1	1 - 15
Y	WO, 87/03602, A1 (Teijin Ltd.), June 18, 1987 (18. 06. 87) & EP, 248909, B1 & US, 5043281, A	9 - 11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
December 28, 1995 (28. 12. 95)	January 30, 1996 (30. 01. 96)

Name and mailing address of the ISA/	Authorized officer
Japanese Patent Office	
Facsimile No.	Telephone No.

国際調査報告

国際出願番号 PCT/JP 95/02427

A. 発明の属する分野の分類（国際特許分類（IPC））

Int. Cl⁶ G01N33/53

B. 調査を行った分野

調査を行った最小限資料（国際特許分類（IPC））

Int. Cl⁶ G01N33/53

最小限資料以外の資料で調査を行った分野に含まれるもの

日本国実用新案公報 1926－1995年
日本国公開実用新案公報 1971－1995年

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）

CAS ON LINE

C. 関連すると認められる文献

引用文献のカテゴリー*	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求の範囲の番号
Y	JP, 64－59068, A（東ソー株式会社）, 6. 3月. 1989（06. 03. 89）（ファミリーなし）	1－15
Y	WO, 94/04700, A1（藤沢薬品工業株式会社）, 3. 3月. 1994（03. 03. 94） &EP, 672756, A1	1－15
A	JP, 63－49095, A（ユニチカ株式会社）, 1. 3月. 1988（01. 03. 88）	1－15

☑ C欄の続きにも文献が列挙されている。

☐ パテントファミリーに関する別紙を参照。

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国際調査を完了した日

28. 12. 95

国際調査報告の発送日

30.01.96

名称及びあて先
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郵便番号100
東京都千代田区霞が関三丁目4番3号

特許庁審査官（権限のある職員） 2J 9408
亀田宏之 印

電話番号 03－3581－1101 内線 3252

C（続き）．関連すると認められる文献

引用文献のカテゴリー＊	引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求の範囲の番号
	＆ＥＰ，２６１７８１，Ａ１	
Y	WO，８７／０３６０２，Ａ１（帝人株式会社），１８．６月．１９８７（１８．０６．８７）＆ＥＰ，２４８９０９，Ｂ１＆US，５０４３２８１，Ａ	９－１１

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Therapeutic Drug Monitoring
15:204–208 © 1993 Raven Press, Ltd., New York

FK506 Measurement: Comparison of Different Analytical Methods

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Summary: In this study, we used solid-phase extraction with Sep-Pak and a liquid–liquid extraction with methylene chloride as two primary methods of extracting FK506 from plasma. The extracts were either analyzed directly by enzyme-linked immunosorbent assay (ELISA) or subjected to high-performance liquid chromatography (HPLC) separation and different fractions were analyzed by ELISA. Serial blood samples were obtained from four kidney transplant patients and four patients who underwent liver transplantation, from day 1 until day 30–35 posttransplantation. There was no significant difference in the FK506 plasma concentration as measured by all four methods in normal transplant patients. However, in liver transplant patients, the solid-phase extraction method gave higher FK506 concentrations than the methylene chloride extraction during the early postoperative period. The concentrations measured after methylene chloride extraction were higher than that after HPLC-ELISA. This higher FK506 concentration measured by direct ELISA could be attributed to possible cross-reacting metabolites that were present in the plasma of patients with abnormal liver functions. Once liver function returns to normal, all four methods give identical plasma concentrations for FK506. **Key Words:** FK506—Immunosuppression.

FK506 is currently under clinical investigation as an immunosuppressive agent in various organ transplant patients (1). It is a very potent compound that appears to have significant nephrotoxicity as a major side effect (2). Pharmacokinetic studies of FK506 have shown that there is a large inter- and intraindividual variation in its kinetics in organ transplant patients (3,4). These factors necessitate routine monitoring of plasma FK506 concentration in transplant patients in an effort to optimize FK506 therapy. Plasma FK506 concentrations are currently measured at the University of Pittsburgh Medical Center by an enzyme-linked immunosorbent assay (ELISA) method after a solid phase extraction (5,6). In addition, FK506 can also be measured in whole blood by a radioreceptor assay (7), high-performance liquid chromatography-mass spectrometry (HPLC-MS) assay (8,9), Abbott IMx method (10), and in serum by a combined HPLC-ELISA method (11). A recent study has shown that FK506 concentrations, as measured by ELISA after a solid phase extraction, are higher than those measured after a liquid–liquid extraction, using methylene chloride (12,13). The primary objective of this study was to evaluate the effect of two different extraction procedures for measurement of trough FK506 concentrations in plasma samples obtained from liver and kidney transplant patients in comparison to a specific analytical procedure that

Received December 11, 1992; accepted February 11, 1993.
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es a HPLC separation step, to separate potential K506 metabolites from FK506 before use of LISA.

MATERIALS AND METHODS

Materials

FK506 drug, monoclonal antibody for FK506, nd FK506 peroxidase enzyme conjugate were supplied by Fujisawa Pharmaceuticals (Osaka, Japan). Anti-mouse IgG was purchased from Atlantic Antibodies (Stillwater, MN, U.S.A.). C-18 Sep-Pak columns were obtained from Waters (Milford, MA, U.S.A.). O-Phenylenediamine (OPD) was purchased from Sigma (St. Louis, MO, U.S.A.). Other reagents were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Methods

Clinical Protocol

Daily trough blood samples were collected from four liver and four kidney transplant patients from day 1 till 30–35 days posttransplantation. Biochemical parameters characterizing kidney function (serum creatinine) and liver function (bilirubin, aspartate aminotransferase, alanine aminotransferase) were measured in these patients over the entire study period. Blood samples obtained for FK506 measurement were incubated at 37° for 45 min. The plasma was separated at 37°C and extracted by the methods described below and subjected to ELISA analysis.

Solid-Phase Extraction

Plasma (100 µl) in duplicate was acidified with 1 ml of 0.1 *N* HCl and passed through a C-18 Sep-Pak cartridge which was prewashed with 4% acetic acid. After an additional wash with 4% acetic acid, FK506 was eluted with 2 ml methanol (6). The methanol was evaporated under nitrogen and the FK506 content was analyzed by ELISA.

Liquid–Liquid Extraction with Methylene Chloride

Plasma (100 µl) in duplicate was acidified with 1 ml of 0.1 *N* HCl and extracted with 5 ml of methylene chloride. This procedure is a minor modification of the procedure described by Kobayashi et al. (12). The organic layer was collected and evaporated under nitrogen at room temperature and FK506 content was analyzed by ELISA.

High-Performance Liquid Chromatography

Plasma extracts prepared by solid phase extraction and methylene chloride extraction were dissolved in methanol and subjected to HPLC separation. HPLC was performed on a Waters 600E multisolvent delivery system equipped with a system controller and a 994 photodiode array detector. For the separation of FK506, its metabolites, and endogenous compounds, we used 3.9-mm ID × 15.0-cm long analytical column filled with µBondaPak C-18 (Catalog no. 86684, Waters Associates). The column temperature was set to 60°C for the analysis. A mobile phase consisting of 80% methanol and 20% H_2O (acidified to pH 6.0 with HCl) was used to elute different components from the column at a flow rate of 0.8 ml/min. The eluents were monitored at 214 nm. The retention time of FK506 was 4.8 min under these conditions. Two fractions were collected from 0–3.6 and 3.6–6 min with greater than 99% of the parent FK506 being collected in the second fraction. Both fractions were evaporated under nitrogen and the residue analyzed for FK506 using ELISA.

RESULTS

The biochemical profiles of the patients studied are listed in Table 1. Patients received intravenous FK506 at a dose of 0.05 mg/kg/day as a continuous

TABLE 1. *Biochemical profile in four liver and four kidney transplant patients*

	Creatinine (mg/dl)		Bilirubin (mg/dl)		AST[a] (IU/L)		ALT[b] (IU/L)	
Patient name	c	d	c	d	c	d	c	d
Kidney								
HB	11.8	1.9	0.4	0.3	31	12	21	51
HG	10.0	2.3	0.4	1.2	51	46	80	86
GW	8.8	3.0	1.3	0.8	20	36	10	56
FB	7.5	1.9	0.5	0.4	15	9	32	20
Liver								
MK	1.8	1.9	2.8	0.9	952	21	1,188	29
JR	1.9	1.4	7.1	0.9	194	11	303	30
BC	0.3	1.3	1.8	0.7	69	49	1,423	61
AA	0.4	0.8	3.1	1.0	59	30	159	31

[a] Aspartate aminotransferase.
[b] Alanine aminotransferase.
[c] During the immediate postoperative period.
[d] Four weeks after transplantation.

infusion during the immediate postoperative time and for up to 3–7 days. They were converted to oral therapy at a dose of 0.15 mg/kg day as soon as they were ready for oral intake. All kidney transplant patients had normal liver function tests and all liver transplant patients had normal kidney function throughout the entire course of study. The interday coefficient of variation of the HPLC-ELISA method is 16.5% (n = 20). Because HPLC-ELISA after Sep-Pak and methylene chloride were similar ($r^2 = 0.95$), these values were averaged and compared with direct ELISA. The regression equation of the FK506 concentrations measured by methylene chloride-ELISA versus Sep-Pak-ELISA is given as follows: methylene chloride method (ng/ml) = 0.066 + 0.91 [Sep-Pak method (ng/ml)], $r^2 = 0.94$; $p < 0.01$.

In all the kidney patients, the concentration of FK506 in plasma was very similar when measured by ELISA following Sep-Pak or methylene chloride extraction as indicated in Fig. 1. In addition, separation of potential FK metabolites from parent FK506 by HPLC and subsequent ELISA assay also gave similar FK506 concentrations. In this group of patients, there was no difference in FK506 concentration as measured by the three methods, irrespective of whether the patient was on intravenous or oral FK506 therapy, or irrespective of the functional status of the kidney.

In liver transplant patients, during the immediate postoperative period, the bilirubin concentration was high and decreased to normal values over a time period, as indicated in Fig. 2. In three of the patients (JR, MK, AA), during the immediate postoperative period, serum bilirubin was elevated, and FK506 concentrations measured by Sep-Pak-ELISA was higher in comparison with methylene chloride-ELISA. However, both of these estimates were also higher than the values obtained by HPLC-ELISA. As the bilirubin concentrations returned toward normal values, the concentration of FK506 measured by all three methods was almost identical. In one patient (BC), however, from day 1 posttransplant all the assay methods provided the same values. The highest total bilirubin in this patient was only 1.8 mg/dl which is indicative of near normal function of the liver. In this patient, on days 10 and 32, FK506 concentrations were significantly elevated as measured by all three methods.

DISCUSSION

Over the past several years, we have been using a Sep-Pak extraction procedure for measuring plasma concentrations of FK506. Recently, a methylene chloride extraction procedure has been reported for measurement of FK506 in plasma (12). This procedure tends to give lower values in comparison to the Sep-Pak extraction procedure. It is believed that this is due to accumulated FK506 metabolites that co-elute in the solid-phase extraction and that are not extracted by the methylene chlo-

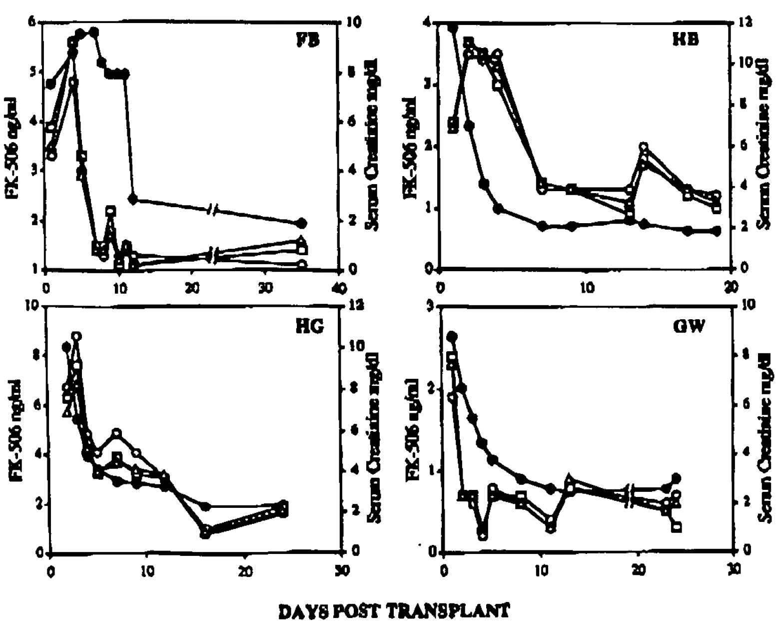

FIG. 1. Plasma FK506 concentrations as measured by Sep-Pak ELISA (○), methylene chloride ELISA (□), HPLC ELISA (△), and creatinine (●) in four kidney transplant patients.

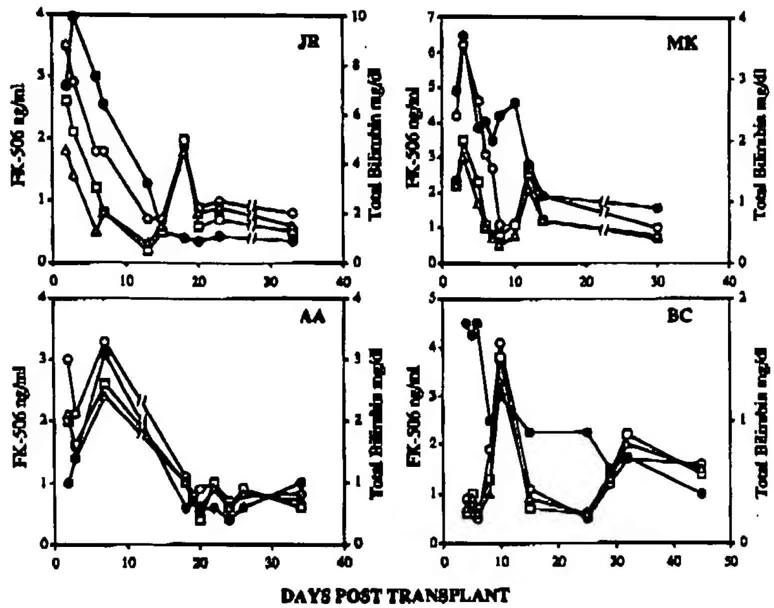

FIG. 2. Plasma FK506 concentrations as measured by Sep-Pak ELISA (○), methylene chloride ELISA (□), HPLC ELISA (△), and bilirubin (●) in four liver transplant patients.

ride. In order to evaluate the specificity of the Sep-Pak and methylene chloride methods, we developed an HPLC-ELISA method for measuring parent FK506 in plasma. Our results indicate that in patients with normal liver function, concentrations of FK506, as measured by a specific HPLC-ELISA method, is similar to the concentrations measured by Sep-Pak-ELISA or methylene chloride-ELISA. This is true independently of whether the organ transplanted is kidney or liver. This is perhaps due to the fact that in patients with normal hepatic function, very little, if any, of the metabolites of FK506 accumulate in the plasma and are, therefore, extracted from plasma. In liver transplant patients, during the immediate postoperative period when the liver function is not normal, as indicated by elevated bilirubin concentrations, the Sep-Pak procedure gives the highest values and HPLC-ELISA gives the lowest, indicating accumulation of the metabolites in these patients. These metabolites cross-react with the monoclonal antibody used in the ELISA procedure. Once hepatic function improves, the liver is able to clear most of the metabolites of FK506 with the result that there is no significant difference between concentrations measured by the different methods.

Our results also indicate that changes in kidney function, as measured by serum creatinine, did not have any influence on the FK506 concentration as measured by all three methods. This suggests that only a very small amount of FK506 metabolites are, in fact, excreted through the kidney. This fact is supported by our previous observation of renal clearance of FK506 to be <1% of total body clearance (4).

The observation that FK506 levels, measured by the three different methods, are also independent of the route of administration, suggest the following: (a) There is no route-dependent metabolism of FK506, indicating minimal gut metabolism; and/or (b) any metabolite produced in the small intestine minimally cross-reacts with the monoclonal antibody used in the ELISA assay. Recent evidence points to the potential for gut metabolism of FK in humans (9). However, the extent of cross reactivity of the metabolites produced in the small intestine with the monoclonal antibody used in ELISA is not known at this time.

CONCLUSIONS

A solid phase extraction method and a liquid–liquid extraction method using methylene chloride were studied, in comparison to an HPLC method for measuring FK506 plasma concentrations. In kidney transplant patients, there was no difference in FK506 concentrations obtained by the different procedures used. In liver transplant patients, the solid-phase extraction method gave higher FK506 concentrations than the methylene chloride extraction only in patients with abnormal liver function. HPLC separation procedure before ELISA indicated that the elevated FK506 levels from solid-phase-ELISA and methylene chloride-ELISA were

due to cross-reacting metabolites that were present in these patients. Presently, at our medical center patients are maintained at trough plasma concentrations between 0.5 and 2.0 ng/ml. Therapeutic range for FK506 concentrations in plasma is independent of the assay method used in patients with normal liver function. However, in the presence of liver dysfunction, both methylene chloride-ELISA and Sep-Pak-ELISA will measure some of the metabolites and this should be kept in mind in interpreting the data.

Acknowledgment: We acknowledge the Pathology Education & Research Foundation (PERF) for their financial support of this project.

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A Highly Sensitive Method to Assay FK-506 Levels in Plasma

K. Tamura, M. Kobayashi, K. Hashimoto, K. Kojima, K. Nagase, K. Iwasaki, T. Kaizu, H. Tanaka, and M. Niwa

FK-506, A MACROLIDE antibiotic obtained from *Streptomyces tsukubaensis*, strain No. 9993, was isolated from the soil of Tsukuba, northern Japan.[1] Its structure has been determined chemically[2,3] and by x-ray crystallography.[4] FK-506 has been shown to have strong immunosuppressive activity against a mixed lymphocyte reaction (MLR)[1] and promises to be useful in organ transplantation.[5] Its mechanism is considered to be a suppression of both interleukin 2 (IL-2) and IL-2 receptor expression on T cells.[1,6] Its activity has been reported to be 100 times more potent than that of cyclosporine A (CsA). As such, a sensitive assay method for a more potent immunosuppressant of FK-506 is crucial for pharmacokinetic studies and for setting effective administration dosages.

Here, we report a simple, safe, and sensitive enzyme immunoassay (EIA) for FK-506. The pharmacokinetics were also monitored after low-dose administration of FK-506 in dogs.

MATERIALS AND METHODS

Preparation of FK-506 Hemisuccinate

FK-506 hemisuccinate (90 mg) was prepared from 248 mg FK-506 and 145 mg succinic anhydride with 4-dimethylaminopyridine in pyridine.

Preparation of Activated Ester of FK-506 Hemisuccinate

The activated ester (74.1 mg) was prepared by reacting 90 mg of FK-506 hemisuccinate and 12.7 mg of *N*-hydroxysuccinimide with dicyclohexylcarbodiimide in ethyl acetate.

Preparation of BSA–FK-506 Conjugate

A solution of bovine serum albumin (BSA) (Armour Pharmaceutical Co, Kankakee, IL; 197 mg) in 50 mmol/L phosphate buffer (PB; 6 mL, pH 7.3) was added to a solution of the activated ester (37 mg) in dioxane (3 mL). The solution was stirred for three days at 4°C and then dialyzed against 50 mmol/L PB (pH 7.3) for 24 hours at 4°C. The preparation was used without further purification for raising antibodies both in the rabbit and mouse.

Preparation of Horseradish Peroxidase–FK-506 Conjugate

An activated ester solution of FK-506 (0.48 mg) in dioxane (10 μL) was mixed with a solution of horseradish peroxidase (POD; type IV, Sigma Chemical Co, St Louis, MO; 10 mg) in 0.18 mL of dioxane and 0.18 mL of 0.5% $NaHCO_3$ solution and stirred at 4°C for 2.5 hours. To this reaction mixture, 1.77 mL of 50 mmol/L PB (pH 7.0) containing 0.1% (wt/vol) gelatine was added, and the solution was dialyzed against 50 mmol/L PB (pH 7.0) completely. This POD–FK-506 solution was diluted 2×10^5-fold with 1% (wt/vol) BSA in phosphate buffered saline (PBS) (1% BSA-PBS) and used in the assay.

Preparation of Polyclonal Antibody to FK-506

The BSA–FK-506 conjugate (1.6 mg as BSA) in PBS solution (2.5 mL) was used to immunize a New Zealand white rabbit with Freund's complete adjuvant (FCA) (2.5 mL) and was followed by a couple of boosters containing a suspension of BSA–FK-506 conjugate (1.6 mg as BSA) in PBS (2.5 mL) and Freund's incomplete adjuvant (FIA) (2.5 mL). Serum with high antibody titers and with suppressive activity to FK-506 MLR inhibition was collected, and a crude IgG fraction was precipitated at 33% $(NH_4)_2SO_4$. IgG was purified on DE-52 columns in 20 mmol/L PB (pH 8.0). Fractions that passed through the column were collected and used for EIA as purified polyclonal antibody (PcAb) against FK-506.

Preparation of Monoclonal Antibody to FK-506

A PBS solution (0.2 mL) of the BSA–FK-506 conjugate (50 μg as BSA) was used to immunize BALB/c mice

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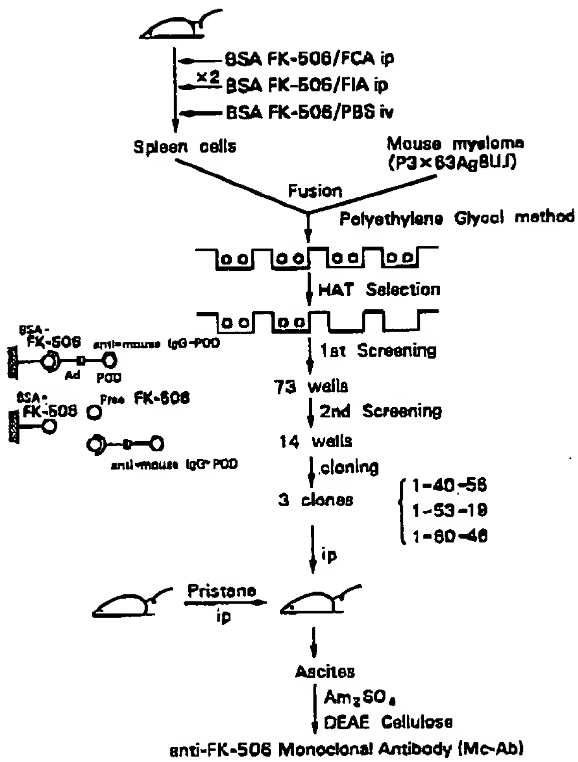

Fig 1. **Preparation of mouse MoAb to FK-506.**

with 0.2 mL of FCA (Fig 1). The mice were boosted a couple of times with intraperitoneal injections of BSA–FK-506 (0.2 mL; 50 μg as BSA) and FIA (0.2 mL). Three days after the last boosting with an intravenous injection of BSA–FK-506 (0.2 mL; 200 μg as BSA), the spleen was excised and used for cell fusion. The spleen cells were fused with mouse myeloma cells (P3X63Ag8U.1) at a 5:1 ratio with 45% polyethylene glycol. After hypoxanthine-aminopterin-thymidine (HAT) selection, hybridoma cells were screened for specific IgG against FK-506 and not against BSA. Clones whose supernatant showed positive reactions in their affinity to BSA–FK-506 but became negative in the presence of excess free FK-506 were selected. After cloning by the limiting dilution method, three clones were expanded in the peritoneal cavity of pristane-primed BALB/c mice, and IgG was purified by $(NH_4)_2SO_4$ precipitation and DE-52 chromatography. Each purified IgG was characterized by the Ouchterlony method, and their detectability for FK-506 was tested in one-step EIA.

Two-Step EIA Procedure

First, antimouse IgG Ab was adsorbed onto a 96-well microtiter plate (Sumitomo Bakelite MS-3596F, Tokyo) by incubating the plate overnight at 4°C with 200 μL of antimouse IgG Ab in PBS (3 μg/mL) per well. The antibody solution was aspirated off, 300 μL of 1% BSA-PBS solution was added, and the plate was incubated for 30 minutes at 37°C to cover all nonspecific binding sites. The solution was substituted with 100 μL of the 2 × 10^5-fold–diluted solution of POD–FK-506 diluted with 1% BSA-PBS. To this solution in the well was added 100 μL of FK-506 solution (FK-506 standard solution appropriately diluted with 10% normal plasma in 1% BSA-PBS or a tenfold-diluted plasma sample in 1% BSA-PBS from an animal treated with FK-506). Then, 50 μL of monoclonal antibody (MoAb) solution (10 ng/mL) in 1% BSA-PBS was added to each well, and the plate was incubated at 4°C overnight. The solution was aspirated off, and the plate was washed with 0.05% Tween 20–PBS and washed again with PBS. The enzyme substrate solution was added, and the plate was left at room temperature for 30 to 60 minutes until a good optical density of 492 nm (OD_{492}) was attained. The colorimetric reaction was stopped by the addition of 50 μL of a 4 N H_2SO_4 aqueous solution. The OD_{492} was measured and plotted on a semilogarithmic graph. The concentration of FK-506 is on the abscissa of the logarithmic scale, and OD_{492} is on the ordinate, of the normal scale.

Enzyme substrate solution was prepared by dissolving 100 mg of *o*-phenylenediamine hydrochloride and 50 μL of 30% H_2O_2 in 100 mL of phosphate-citrate buffer (pH 5.4). The phosphate-citrate buffer (pH 5.4) used in this experiment was prepared by adjusting the pH of a 0.1 mol/L Na_2HPO_4 aqueous solution with a 0.1 mol/L citric acid aqueous solution to pH 5.4.

One-Step EIA Procedure

The PcAb or MoAb concentration used in one-step EIA was 20 μg/mL in PBS. Competitive reaction between free FK-506 and POD–FK-506 was started by the addition of these two components. The concentration of POD–FK-506 and the rest of the procedure were the same as those in two-step EIA (Fig 2).

RESULTS

Preparation of PcAb to FK-506

After the fourth immunization, the titer (the dilution fold that causes 50% decrease of antibody binding to BSA-FK-506) was 10^4. From 340 mL of antiserum, 5,646 mg of PcAb to FK-506 was obtained by purification as described in Materials and Methods.

Preparation of MoAb to FK-506

After HAT selection and two screenings, three hybridomas that produced MoAbs specific against FK-506 were cloned. Each of these monoclonal hybridomas, 1-40-56, 1-53-19, and 1-60-46, was expanded in the perito-

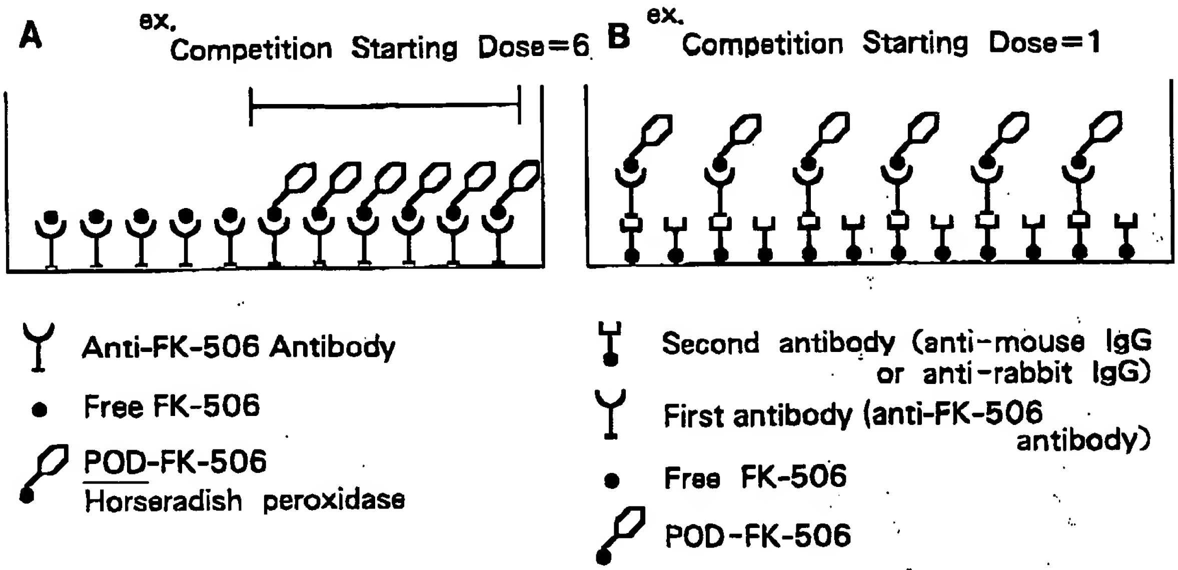

Fig 2. Illustration of (A) one-step EIA and (B) two-step EIA.

neal cavity of pristane-primed BALB/c mice and yielded respective purified anti–FK-506 MoAbs after conventional purification. The data of MoAb production and their characteristics are shown in Table 1.

Comparison of One-Step EIA With PcAb and MoAb

The results of one-step EIA with PcAbs and MoAbs are shown in Fig 3. PcAb gave the highest sensitivity, and the minimum detection level was 0.33 ng/mL in 10% plasma. Of the MoAbs, MoAb 1-60-46 gave the best sensitivity, which was 1 ng/mL in 10% plasma. The most striking difference between MoAb 1-60-46 and PcAb was that the standard curve was steeper with the MoAb, with a detection range from 1 ng/mL in 10% plasma to 100 ng/mL in 10% plasma, whereas that with PcAb was shallow and gave a wider detection range from 0.33 ng/mL in 10% plasma to 10^3 ng/mL in 10% plasma.

Table 1. MoAb Production and Their Subclass

MoAb	Ascites (mL)	Purified IgG (mg)	IgG Subclass
1-40-56	11	125.5	IgG1
1-53-19	6	18.7	IgG2b
1-60-46	40	175.0	IgG1

Precision of One-Step EIA Using PcAb

Intraassay and interassay variances were assessed from the data with appropriately diluted FK-506 in 10% plasma and are shown in Table 2. The coefficients of variance were 5.8% to 24.5% for intraassay and 4.0% to 11.5% for interassay.

Effect of Plasma Concentration on the Assay and Elimination of the Effect by Benzene Extraction

The EIA sensitivity was found to depend heavily on the plasma concentration (Fig 4). When FK-506 in 100% plasma was diluted tenfold, the minimum detectable level was almost the same as that in 1% BSA-PBS, but this means that the sensitivity was lowered by tenfold. This interference by plasma concentrations over 10% was eliminated by benzene extraction of the plasma sample (Fig 4); dichloromethane extraction could also eliminate the effect (data not shown).

More Highly Sensitive Assay System of Two-Step EIA

When the two-step EIA was applied to PcAb, the minimum detectable level came down to 1 pg/mL, a 300-fold improvement in

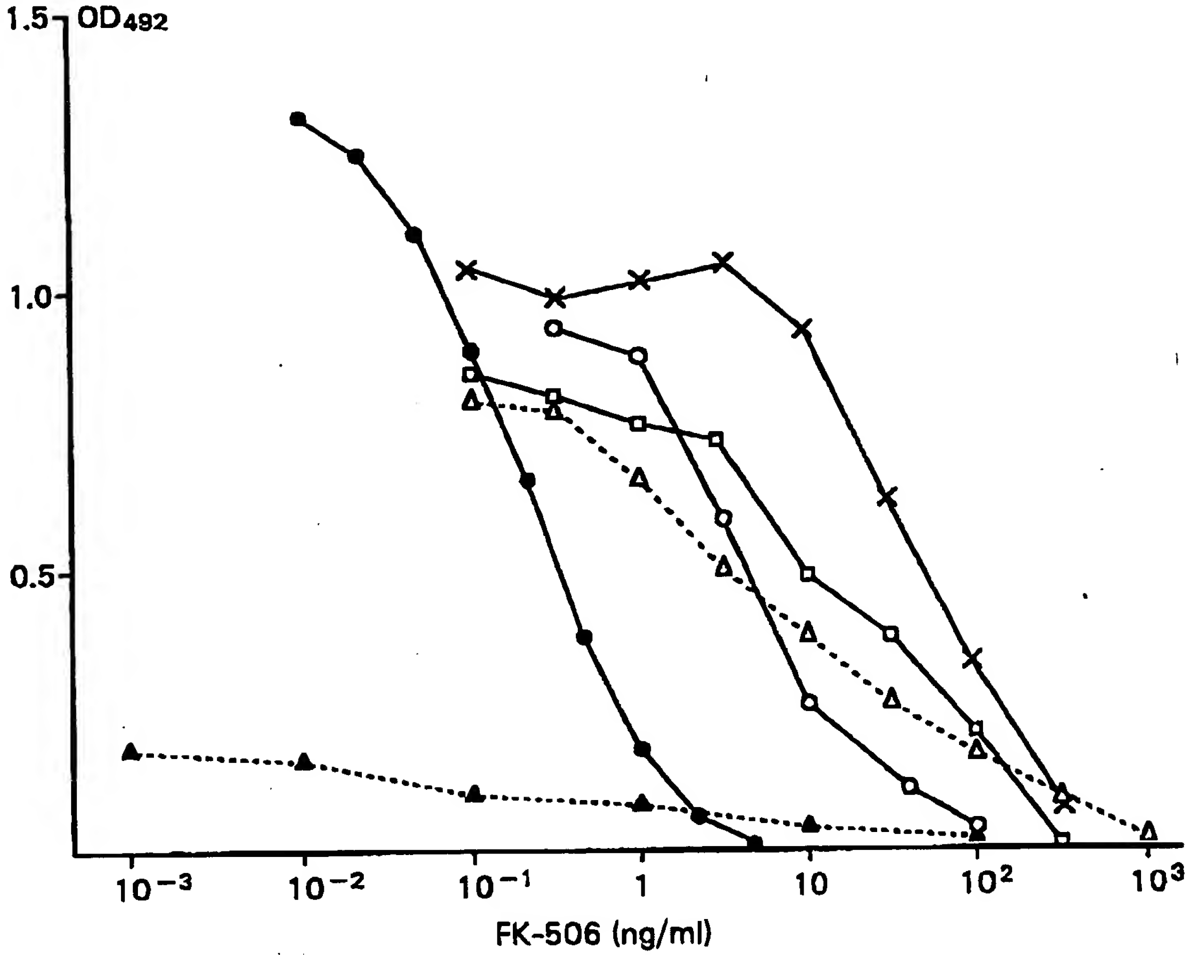

Fig 3. Standard curves of FK-506 by two-step EIA using PcAb and MoAb 1-60-46 and those by one-step EIA using PcAb and three MoAbs. △----△, PcAb one-step EIA; X—X, MoAb 1-40-56 one-step EIA; □—□, MoAb 1-63-19 one-step EIA; O—O, MoAb 1-60-46 one-step EIA; ▲----▲, PcAb two-step EIA; ●—●, MoAb 1-60-46 two-step EIA.

the sensitivity. Next, MoAb 1-60-46 was examined for its applicability to this two-step EIA. As a second Ab, rabbit PcAb to mouse IgG prepared in our laboratory was used. In this assay with MoAb 1-60-46, a good minimum detectable level of 20 pg/mL in 10% plasma was obtained. Again, the calibration curve for standard FK-506 in 10% plasma was steeper than that with PcAb (Fig 3).

Precision of Two-Step EIA With MoAbs

The intraassay and interassay coefficients of variance were 6.0% to 11.7% and 4.9% to 23.1%, respectively (Table 3).

Table 2. Precision of PcAb One-Step EIA: Coefficients of Variance for FK-506 in 10% Dog Plasma

Sample	Intraassay (n = 7)		Interassay (n = 4)	
	Mean ± SD (ng/mL)	CV (%)	Mean ± SD (ng/mL)	CV (%)
A	0.388 ± 0.0517	13.4	0.389 ± 0.0448	11.5
B	2.670 ± 0.4619	17.3	2.630 ± 0.1578	6.0
C	23.9 ± 5.858	24.5	22.7 ± 0.9032	4.0
D	80.5 ± 4.650	5.8	81.4 ± 4.540	5.6

Abbreviation: CV, coefficient of variance.

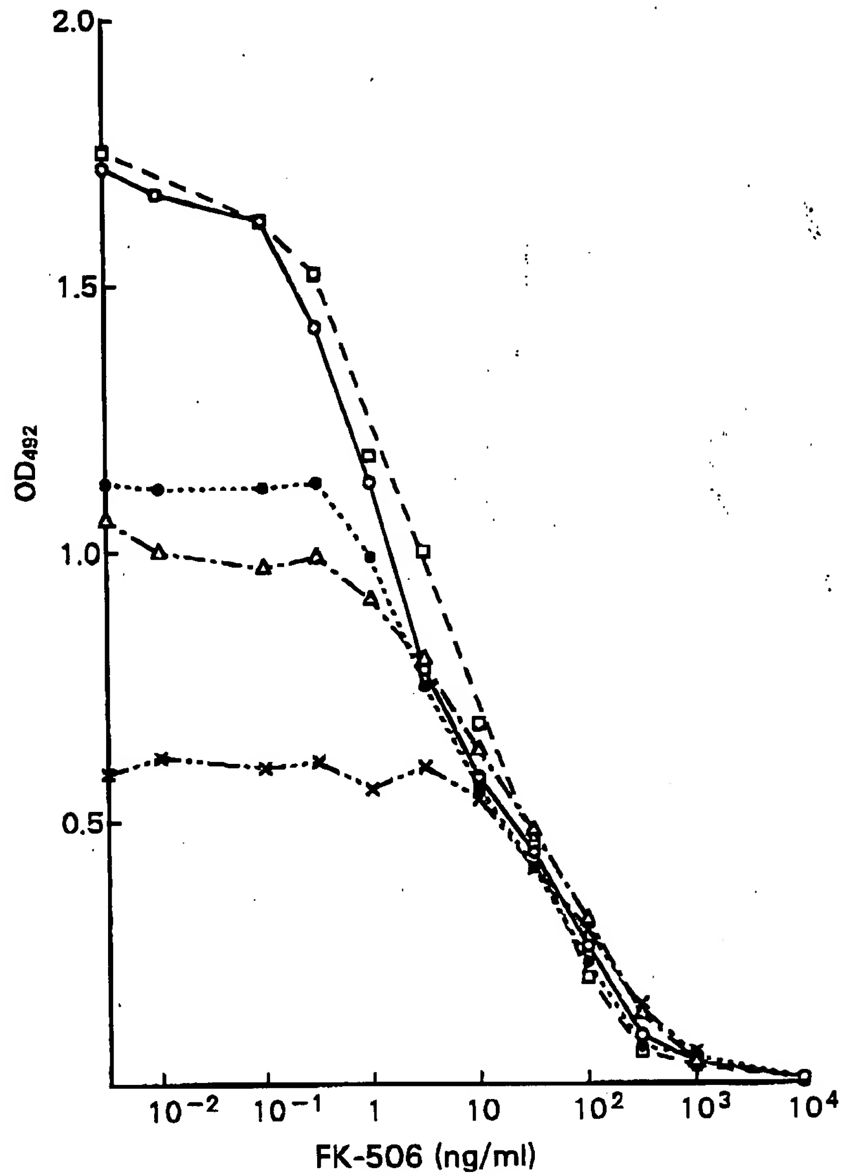

Fig 4. Influence of plasma concentration on the one-step EIA of FK-506 and elimination of the influence by benzene extraction. ○—○, FK-506 in 1% BSA-PBS; ●—●, FK-506 in 10% dog plasma 1% BSA-PBS; △-—-△, FK-506 in 20% dog plasma 1% BSA-PBS; X———X, FK-506 in 50% dog plasma 1% BSA-PBS; □———□, benzene extraction from plasma.

Pharmacokinetics of FK-506 in Dogs After Oral Administration—Sensitive Two-Step EIA

The plasma concentration of FK-506 after 0.32 or 1 mg/kg administration of oral formulation (solid dispersion formulation [SDF][7]) was measured by two-step EIA after extraction pretreatment and was shown in Fig 5. In these cases, the plasma concentration of FK-506 could not be measured by one-step EIA. Oral administration gave a peak of FK-506

Table 3. Precision of MoAb Two-Step EIA: Coefficients of Variance for FK-506 in 10% Dog Plasma

	Intraassay (n = 6)		Interassay (n = 3)	
Sample	Mean ± SD (ng/mL)	CV (%)	Mean ± SD (ng/mL)	CV (%)
A	0.040 ± 0.0039	9.8	0.042 ± 0.0097	23.1
B	0.119 ± 0.0140	11.7	0.126 ± 0.0087	6.9
C	0.642 ± 0.0408	6.4	0.668 ± 0.0327	4.9
D	2.49 ± 0.1484	6.0	2.31 ± 0.3350	14.5

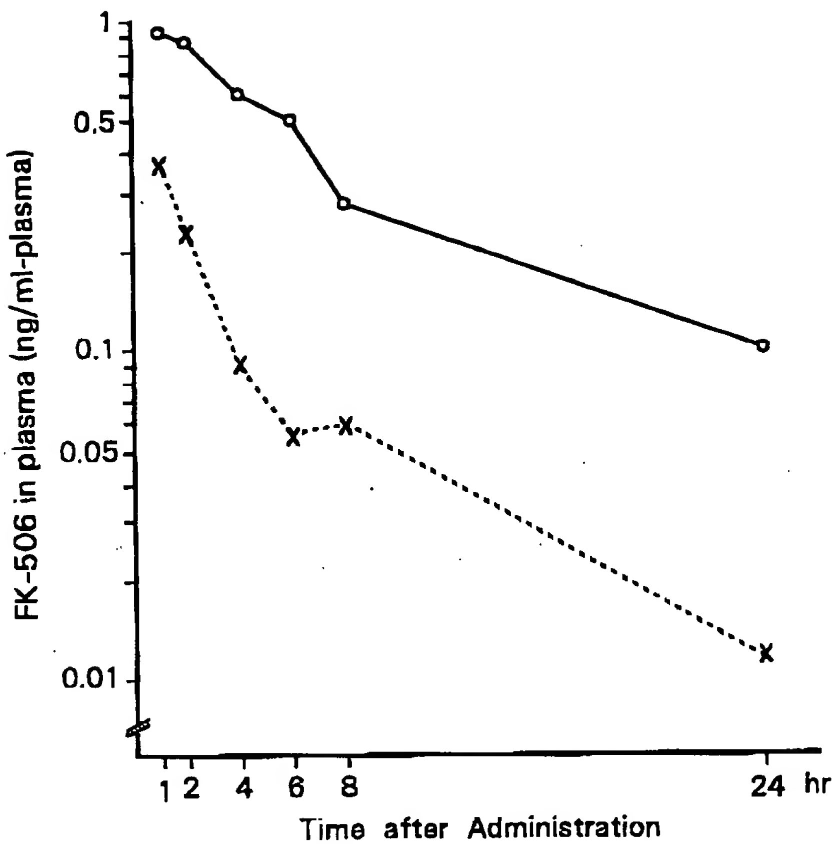

Fig 5. Pharmacokinetics of FK-506 following bolus oral administration of FK-506. X------X, 0.32 mg/kg; O——O, 1 mg/kg.

within one hour after administration of either dosage, and at 24 hours, the FK-506 concentration was 10 pg/mL at a dose of 0.32 mg/kg and 100 pg/mL at 1 mg/kg.

DISCUSSION

Rabbit PcAb and three mouse MoAbs against FK-506 were prepared by using BSA–FK-506 as a hapten-carrier antigen. By using these antibodies, two EIA systems were established: one-step EIA and two-step EIA (Fig 2).

Anti–FK-506 Ab is adsorbed onto the plate first in one-step EIA, and the second Ab of antimouse IgG or antirabbit IgG is adsorbed onto the plate first in two-step EIA. As a result, we have four combinations of Abs and assay systems: PcAb one-step EIA, PcAb two-step EIA, MoAb one-step EIA, and MoAb two-step EIA. Of three MoAbs, MoAb 1-60-46 gave the best sensitivity in one-step EIA (Fig 3) and was chosen for further study. But even with this MoAb, the sensitivity (1 ng/mL in 10% plasma) in one-step EIA was inferior to that of PcAb (0.33 ng/mL in 10% plasma). This PcAb one-step EIA method is rather simple and has satisfactory intraassay (5.8% to 24.5%) and interassay (4.0% to 11.5%) coefficients of variance when the wide detection range of 0.33 to 10^3 ng/mL in 10% plasma was taken into account, but it was still not sensitive enough to monitor the plasma FK-506 concentration at effective doses in dogs. When the mechanism of EIA is considered, higher sensitivity can be expected with lower concentrations of POD–FK-506. But if the number of anti–FK-506 Abs adsorbed on the plate is large compared with that of POD–FK-506, the free FK-506 might occupy the extra open anti–FK-506 Ab (Fig 2). In this sense, the smaller the number of the bound anti–FK-506 Ab (either PcAb or MoAb), the higher the sensitivity of the EIA. But it is difficult to reduce the number of bound anti–

FK-506 Ab on the plate accurately by only reducing the concentration ratio of the Ab by mixing with another protein such as BSA (data not shown). Thus, we tried to lower the number of anti–FK-506 Ab bound on the plate by using a second Ab to anti–FK-506 Ab. When a combination of antirabbit IgG Ab and anti–FK-506 PcAb (rabbit) was used, the minimum detectable level rose to 1 pg/mL in 10% plasma (Fig 3). When the combination of antimouse IgG Ab and anti–FK-506 MoAb was used, the minimum detectable level rose to 20 pg/mL in 10% plasma. As is evident from Fig 3, the maximum OD_{492} was as low as 0.2 for the former combination, whereas the maximum OD_{492} for the latter combination was the normal level of 1.5. Because it was considered that, when the maximum OD_{492} is low, slight errors in reading the OD_{492} might cause a larger error in FK-506 concentration, we concluded that a combination of anti–FK-506 MoAb and antimouse IgG Ab was the one to be adopted for two-step EIA. The minimum detectable level of 20 pg/mL is a 16-fold improvement over the 0.33 ng/mL by PcAb one-step EIA and a 50-fold improvement over the 1 ng/mL of MoAb one-step EIA (Table 4).

The new method of two-step EIA afforded a highly sensitive assay method. This technique is considered not to be limited only to FK-506 but can be applied to any enzyme immunoassay that requires very high sensitivity. By using this highly sensitive EIA, we measured the plasma concentration of FK-506 after 0.32 and 1 mg/kg oral administration (SDF). As shown in Fig 5, the FK-506 plasma concentration was clearly monitored after bolus administration. With 0.32 mg/kg orally (SDF), a peak plasma concentration (0.4 ng/mL) was attained one hour after administration, and a concentration of 0.09 to 0.06 ng/mL was maintained from four to eight hours, whereas with 1 mg/kg orally (SDF), the plasma peak level was 0.9 ng/mL one hour after administration, and concentrations of 0.6 to 0.3 ng/mL were maintained from four to eight hours.

Here we have shown a highly sensitive new two-step EIA and that the pharmacokinetics of low-dose FK-506 can be monitored. The best dose form and route of FK-506 administration should be determined in accord with its pharmacokinetics.

Table 4. Summary of Minimum Detection Level

Assay	Antibody	Sample Pretreatment: Plasma Direct Method	Sample Pretreatment: Extraction Method
One-step EIA	MoAB	10 ng/mL	—
	PcAb	3.3 ng/mL	330 pg/mL
Two-step EIA	MoAb	200 pg/mL	20 pg/mL
	PcAb	10 pg/mL	1 pg/mL

Values were expressed in concentrations of dog plasma.

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A Combined HPLC-ELISA Evaluation of FK 506 in Transplant Patients

M.C. Friob, A. Hassoun, D. Latinne, G. Lhoëst, J.B. Otte, and P.E. Wallemacq

FK 506 is a new immunosuppressant from Fujisawa Pharmaceuticals (Osaka, Japan) currently under clinical trials in organ transplantation. However, besides its recognized therapeutic properties, it does not seem devoid of side effects; hence, it appears advisable to monitor its serum or whole blood concentrations to adjust the dosage regimen. An enzyme-linked immunosorbent assay (ELISA) is currently the method developed by Fujisawa to analyze FK 506 in biologic fluids.

The aim of our study is to evaluate the specificity of the ELISA in hepatic transplant patients, and to develop a method to determine unchanged FK 506 level as well as some of its eventual metabolites. For this purpose, a high performance liquid chromatography (HPLC) system has been developed to separate all the compounds extracted by solid phase, prior to testing on one hand by ELISA and on the other hand in mixed lymphocyte reactions (MLR), to evaluate their eventual cross-reactivity with the monoclonal antibody (MAb) used in the ELISA and or their eventual immunosuppressive properties.

MATERIALS AND METHODS

HPLC

The HPLC isocratic system consisted of a ternary pump (Varian Model 9010), a variable wavelength ultraviolet (UV) detector (Varian Model 9050) connected to an integrator (Varian Model 4400). A column/valve mounting module was used fastened directly to the side of the pump, with a Rheodyne valve and a 20-μL injection loop. The analytical column consisted of a reverse phase Micro-Pak MCH-5 15 cm column heated at 72°C. The mobile phase consisted of a mixture of acetonitrile/water (71/29), and the flow rate was adjusted to 1 mL/min. FK 506 was detected at 212 nm (UV detector sensitivity range set at 0.005 absorbance unit at full scale), with a sensitivity limit evaluated at 25 ng injected.

FK 506 tautomers elute at 4.6 and 5.2 minutes, with a proportion of peak areas of 1:9 (Fig 1). The dry residues obtained after solid phase extraction of 200 μL of serum were reconstituted with 30 μL of methanol, before being injected in the HPLC system. Thirty-second or 1-minute fractions were collected at the outlet of the HPLC during a 20-minute chromatographic run for further ELISA or MLR evaluations, respectively.

ELISA

The ELISA reagents (pure FK 506, MAb anti-FK 506, polyclonal antibody, and FK 506-peroxidase) were kindly provided by Fujisawa. The assay was performed according to the method described elsewhere.[1] C18 solid phase Bond-Elut columns (Analytichem International) were used to extract FK 506 from serum. All measurements were performed in duplicate. The sensitivity limit reaches concentrations as low as 0.1 ng/mL. Within-run and between-run coefficients of variation have been evaluated in the ranges of 9% to 12% and 23% to 29%, respectively.

MLR

The dry residues obtained from the 1-minute collected fractions were first resuspended in 20 μL ethanol 75%, then diluted in the enriched RPMI. Mononuclear cells were isolated from human peripheral blood by density gradient centrifugation on Ficoll-Hypaque medium (d = 1.077). After washing, the cells were suspended in the enriched RPMI medium at a concentration of 10^6 cells/mL. Incubation of 10^5 cells (100 μL) in microplates at 37°C in a 5% CO2 humidified atmosphere, with 100 μL of the reconstituted HPLC fractions, was started after addition of 100 μL of 10^5 nonirradiated allogeneic cells. Each test was performed in triplicate and compared with a normal response to the stimulation. After 5 days incubation, 10 μL of a diluted 1:2 ^{3}H-thymidine solution was added. The incorporation was determined by liquid scintillation counting after an additional 24 hours incubation.

Serum Specimen

A population of 13 pediatric liver transplant recipients was treated with FK 506 on a compassionate basis.[2] Their serum levels were followed regularly by ELISA, and were evaluated, at least once, by the combined procedure HPLC-ELISA. As controls, blank serum and serum spiked with a known amount of FK 506 were used.

RESULTS AND DISCUSSION

All extracted and injected serum specimens were separated in 40 fractions, analyzed subsequently by ELISA, resulting in a profile similar to a chromatogram (Fig 1). The presence of both FK 506 tautomers detected in the UV was confirmed by ELISA at the same times (4.6 and 5.2 minutes) and the same proportion (1:9). However, the injection of some patient's serum displayed, besides the FK 506, the presence of a characteristic peak eluted around 2 minutes in HPLC and cross-reacting significantly with the MAb used in the ELISA (Fig 1). This cross-reacting compound (X) has never been observed in either

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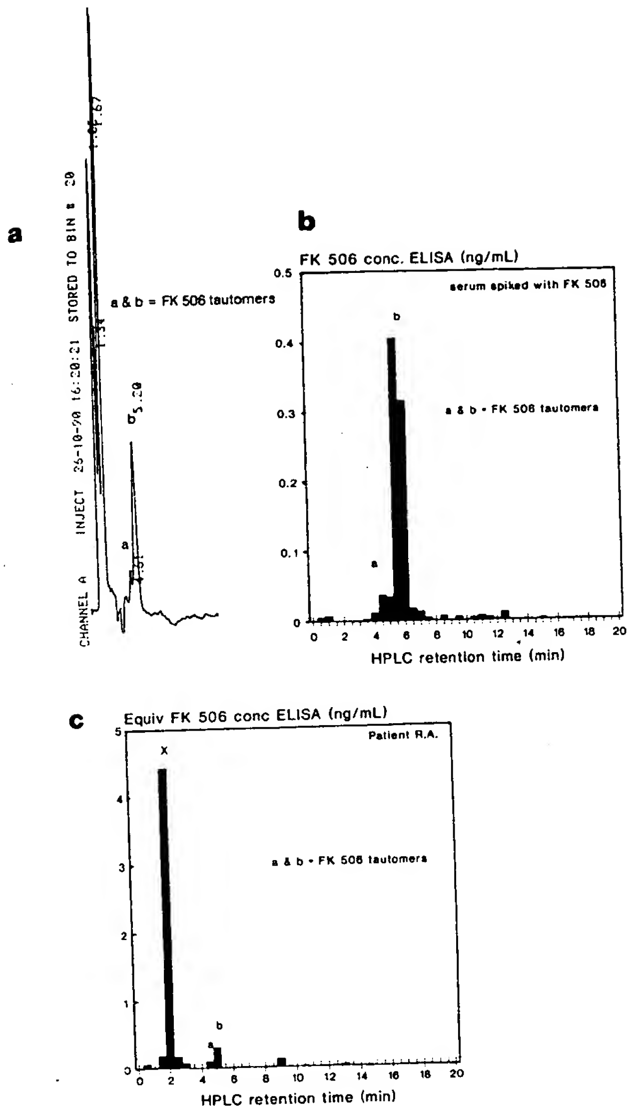

Fig 1. **(a)** HPLC chromatogram obtained after injection of 250 ng of FK 506. FK 506 elutes as two tautomers labeled a and b, detected at 212 nm. **(b)** ELISA profile obtained after HPLC separation of an extracted serum specimen spiked with FK 506 (1 ng/mL). Both tautomers labeled a and b appear at the same retention times than in UV. **(c)** ELISA profile obtained after HPLC separation of an extracted patient's serum. Besides the two tautomers, compound X eluting around 2 minutes cross-reacts significantly with the antibody used in the ELISA.

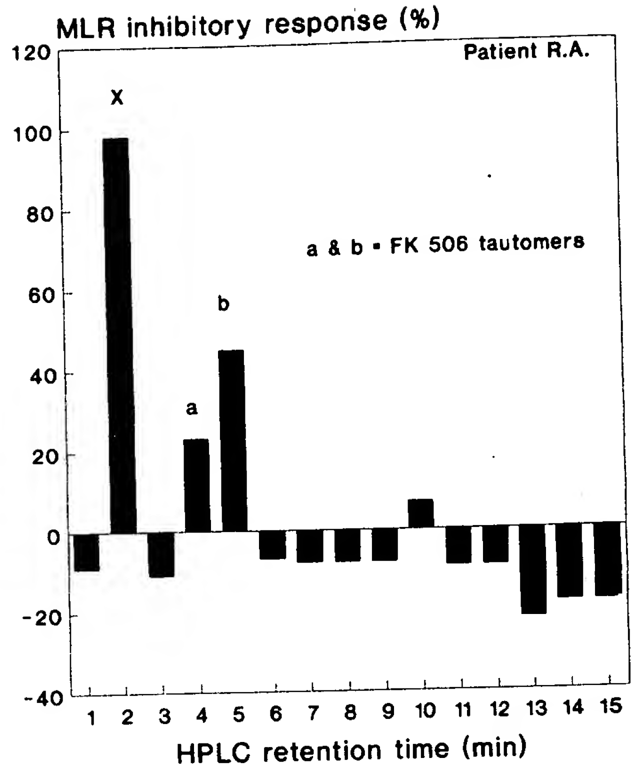

Fig 2. In vitro immunosuppressive profile (MLR) observed after HPLC separation of an extracted patient's serum. A significant immunosuppression is found in the fraction corresponding to the compound X.

blank serum or in serum spiked with FK 506, and was present in several patients at different levels (ratio FK 506:X ranging from 1:0 to 1:10!).

The in vitro study (MLR) of the immunosuppressive activity found in each of the 1-minute collected fractions is illustrated in Fig 2. These data were obtained from a patient serum presenting a ratio FK 506:X of 1:10. Once again the presence of both FK 506 tautomers is confirmed by the immunosuppressive activity found in the corresponding fractions. But, moreover, the compound X clearly displays a significant immunosuppression.

In an attempt to identify the cross-reacting compound X, several concomitant immunosuppressants have been tested in ELISA without presenting any significant interference (cyclosporine in the range 200-400 ng/mL, methylprednisolone, OKT3, azathioprine, anti-T-lymphocyte globulin). It would thus seem likely that the immunosuppressive compound X might be related to FK 506, being a more polar active metabolite (oxidized or conjugate derivative, or another tautomeric form of the drug appearing under certain circumstances). A FAB mass spectrum (Kratos MS80RFA mass spectrometer) of the corresponding eluted fraction has been tentatively studied. Its interpretation remains uncertain due to the very small amount available. However, among others, a peak of 836 mass units (m.u.) is observed and could correspond to a dihydroxylated derivative corroborating the hypothesis of a metabolite. Further studies are currently being undertaken to elucidate this structure.

CONCLUSIONS

The specificity of the ELISA has been studied by combining it with an HPLC system, resulting in a cross-reactivity

profile. Besides, FK 506, a compound coextracted on solid phase, displayed a significant cross-reactivity with the antibody, and an immunosuppressive property. This compound appearing in several patient's serum is thought to be a FK 506 metabolite. The exact role played by this compound X in the immunosuppression or the toxicity of the treatment should obviously be defined in order to understand its involvement in the clinical success of FK 506.

ACKNOWLEDGMENTS

The authors thank Fujisawa Pharmaceuticals for providing FK 506 and ELISA kits.

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Pharmacokinetics of tacrolimus in liver transplant patients

Objective: To characterize the pharmacokinetics of the immunosuppressive agent tacrolimus (FK 506) in liver transplant patients.
Methods: Patients (n = 16) were assessed during and after 1- to 3-day intravenous infusions followed by a 2-week course of oral dose therapy. Plasma and whole blood data were fitted simultaneously with equations accounting for nonlinear drug binding by red blood cells to generate clearance (CL) and volume of distribution (V).
Results: The maximum blood/plasma ratio of tacrolimus was 55.5 ± 26.8 (SD) and half-life averaged 12.1 ± 4.7 hours. The CL and V were relatively high based on plasma concentrations (CL, 1.7 L/hr/kg; V, 30 L/kg) and low based on whole blood (CL, 54 ml/hr/kg; V, 0.9 L/kg), with moderate variability (coefficient of variation, 34% to 49%) among the patients. Correlations of plasma CL and V with maximum blood/plasma ratios (ranging from 13 to 114) were strong (r = 0.65 and r = 0.73). Blood binding affects the disposition of tacrolimus, and plasma concentrations are indirectly and inversely related to red cell binding. The oral dose data for tacrolimus yielded a brief absorption lag time (t_{lag}, 0.39 hour), a variable first-order absorption rate constant (k_a, 4.5 ± 3.0 hr^{-1}), and consistent bioavailability (F, 25% ± 10%). The area under the concentration–time curve versus 12-hour minimum concentration relationships for both whole blood and plasma were nearly linear, confirming the utility of trough values for monitoring drug exposure.
Conclusion: This study provides pharmacokinetic guidelines for the use of tacrolimus in patients undergoing hepatic transplantation. Nonlinear blood binding is a major source of interpatient variation in the disposition of tacrolimus. (Clin Pharmacol Ther 1995;57:281-90.)

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Supported by the Fujisawa Pharmaceutical Company, Deerfield, Ill.

Presented at the meeting of the American Society for Clinical Pharmacology and Therapeutics, Honolulu, Hawaii, March 24–26, 1993 (Abstract: Clin Pharmacol Ther 1993;53:181).

Received for publication June 24, 1994; accepted Sept. 14, 1994.

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0009-9236/95/$3.00 + 0 **13/1/60625**

Tacrolimus (FK 506) is an immunosuppressive agent that is being evaluated for hepatic, renal, and various other types of organ transplantation.[1-3] The compound is more potent than cyclosporine but acts in a similar way by inhibiting signal transduction pathways that lead to T-lymphocyte activation.[4]

The disposition of tacrolimus has been partly assessed in liver transplant patients.[5,6] Venkataraman et al.[5] found, on the basis of drug concentrations in plasma, that tacrolimus has a moderate half-life ($t_{1/2}$) of about 9 hours and appears to have a large volume of distribution, high clearance, and low systemic availability. However, tacrolimus exhibits extensive distribution into red blood cells, and the whole blood to plasma ratio ranges are greater than 30 to 10 over low to high plasma concentrations.[7,8] Current clinical protocols call for monitoring both whole blood and

Table I. Summary of patient characteristics and dosages for tacrolimus studies

Characteristic	*Range*
No. of patients	16
Gender	
Men	9
Women	7
Age (yr)	33-35
Body weight (kg)	53-107
Total infusion dose (mg)	2.7-21.6
Duration of infusion (hr)	18.6-96.1
Oral doses (mg/12 hr)	4-12

plasma concentrations, and uncertainty exists regarding which medium best serves for therapeutic monitoring and pharmacokinetic purposes.[3,8]

This study was carried out as an extension of a randomized trial of tacrolimus versus cyclosporine as primary immunosuppressive treatment in liver transplantation.[3] The purpose was to assess the clinical pharmacokinetics of tacrolimus during infusion therapy over the first 96 hours after transplantation, followed by oral dose therapy for up to 14 days. Examination of disposition of the drug as related to whole blood versus plasma concentrations and characterization of the absorption kinetics of tacrolimus were the aims of this study.

EXPERIMENTAL METHODS

Patients and posology. The study population consisted of 16 adult patients who were undergoing hepatic transplantation at three clinical centers. The general characteristics of the group are provided in Table I. Each patient received an initial infusion of 0.1667 to 0.3077 mg/hr tacrolimus for a period of 31 to 84 hours. Several patients who had been given the higher doses had the infusion rate decreased after 26 to 48 hours. There was usually a 12-hour partial washout phase before the oral dose regimen began at 48 to 230 hours. The oral doses were usually 4 to 12 mg every 12 hours, with later adjustments. Oral dosing was continued so that the last study dose was given at 176.5 to 408 hours after surgery.

Blood samples were collected at designated 1- to 4-hour intervals during and after the infusion of tacrolimus, yielding 7 to 24 pairs of samples. During oral dosing, 10 blood samples were collected at 1- to 2-hour intervals during one to three individual 12-hour oral dosing phases. Samples were also collected at 12-hour times (trough, C_{min}) before each oral dose. These blood samples were maintained at 37° C for 30 minutes before centrifugation at 37° C. Aliquots of whole blood and plasma were then maintained at −20° C before analysis.

Analytic methods. Tacrolimus in plasma and whole blood was measured by use of the enzyme immunoassay of Tamura et al.[9] as adapted by Jusko and D'Ambrosio.[7] The sensitivity and variability of the method have been described previously.[7] Samples from each patient were analyzed on a batch basis.

Whole blood/plasma ratio. The blood/plasma ratio (BPR) of tacrolimus concentrations for each individual patient were fitted by nonlinear least-squares regression (PCNONLIN, SCI Software Inc., Lexington, Ky.) with a previously described equation[10] to obtain the binding capacity (B_{max}) and affinity constant (K_D):

$$BPR = 1 + Hct \cdot \frac{B_{max}}{K_D + C_P} \quad (1)$$

in which Hct is hematocrit and C_p is plasma concentration.

Pharmacokinetics: infusion phase. The first phase of the data analysis involved characterization of the rise and fall of plasma (C_p) and whole blood (C_B) concentrations of tacrolimus during the infusions of the drug over the first 1 to 4 days of therapy. Equations for a one-compartment model with volumes (related to plasma or whole blood), V_p or V_B, and nonlinear binding with red blood cells were adequate to describe the time-pattern of tacrolimus. The following differential equations were used simultaneously:

$$\frac{dC_P}{dt} = \frac{k_o}{V_p} - k_{el} \cdot C_p \quad (2)$$

and

$$\frac{dC_B}{dt} = BPR \cdot \frac{k_o}{V_p} - k_{el} \cdot C_B \quad (3)$$

in which k_o is the infusion rate, k_{el} is the elimination constant ($= CL_p/V_p$ or CL_B/V_B), and CL_p and CL_B are plasma and blood clearance. Weighting of the data was done using the reciprocal of C_p or C_B. Differential, rather than integrated, equations were used because of the nonlinear blood/plasma ratio function (equation 1) and because the adjustments or cessation of infusion rates made during the course of therapy were most easily handled as a direct change in k_o at the appropriate times.

Least-squares values of CL_p and V_p were obtained by use of the PCNONLIN regression program. From these fitted parameters the k_{el} value and the following additional parameters were generated:

Disposition half-life: $t_{1/2} = 0.693/k_{el}$ (4)

Clearance (whole blood): $CL_B = Dose/AUC_B$ (5)

Volume of distribution (whole blood): $V_B = CL_B/k_{el}$ (6)

The clearances and volumes were normalized for total body weight of each patient.

Oral dose phase. The plasma and whole blood concentrations of tacrolimus were fitted to the following equation:

$$C = C^o \cdot e^{-k_{el} \cdot t} + \frac{k_a \cdot D \cdot F}{V(k_{el} - k_a)} (e^{-k_a \cdot t_s} - e^{-k_{el} \cdot t_s}) \quad (7)$$

in which C represents either the plasma (C_p) or whole blood (C_B) concentration of tacrolimus, C^o is the observed initial concentration at the start of the dosing interval, t is the time elapsed since C^o, t_S is t minus t_{lag} where the latter is a lag-time before absorption starts, k_{el} is the elimination constant of the drug (CL_p/V_p or CL_B/V_B), k_a is the first-order absorption rate constant, D is the oral dose, F is the bioavailability, and V is either plasma volume (V_p) or whole blood volume of distribution (V_B). Because the dosing interval is 12 hours for a drug with a $t_{1/2}$ averaging 12 hours, it was necessary to use the k_{el} value obtained from the intravenous infusion phase. Also, the nonlinear function (equation 1) for red blood cell binding of tacrolimus was applied. This left k_a, t_{lag}, and F as parameters to be generated by least-squares regression. Equation 7 was used with the appropriate C_p^o or C_B^o initial value to simultaneously fitted the plasma and whole blood concentrations. These equations do not require steady-state conditions and characterize the kinetics of the current dose of drug with the second term while accounting for residual drug with the first term. The value of k_a was given an upper limit of 8 hr^{-1}. The PCNONLIN program was used for fitting the data.

The calculated value of F based on plasma (F_p) and whole blood (F_B) concentrations was also obtained by determination of the area under the curve over 0 to 12 hours (AUC) by trapezoidal integration. Bioavailability values were obtained from the following:

$$F_p = AUC_p \cdot CL_p/D \quad (8)$$

$$F_B = AUC_B \cdot CL_B/D \cdot BPR \quad (9)$$

in which the CL value is that from the infusion phase. The calculated F was then obtained as the average of F_p and F_B.

Both methods of obtaining F (fitted and calculated) require the assumption that the disposition kinetics of tacrolimus are stationary, allowing use of the CL or k_{el} values from the infusion phase to apply to the oral dose phase. Also, the use of equations 8 and 9 assumes steady-state conditions during oral dosing.

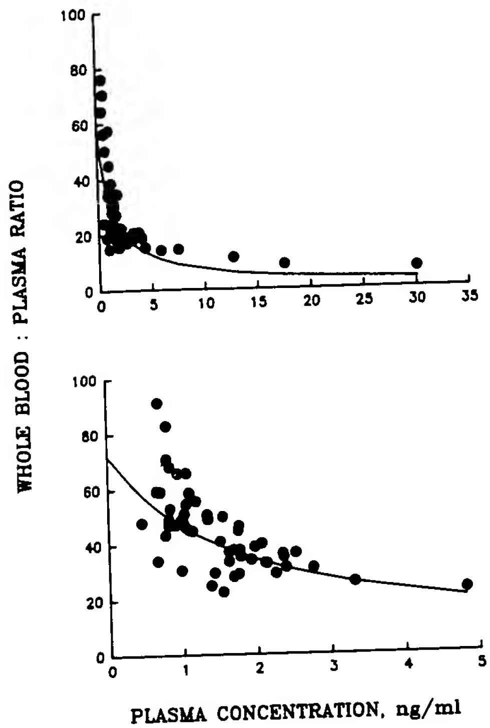

Fig. 1. Relationship between whole blood/plasma ratio and plasma concentration in two patients. *Circles* are experimental data, and *lines* show least-squares fitting of data to equation 1.

Statistics. Regression analyses for which pharmacokinetic parameters were interrelated involved use of the perpendicular least-squares method of Riggs et al.[11]

The relationship between AUC and C_{min} was tested for nonlinearity with PCNONLIN by fitting the relationship:

$$AUC = a \cdot C_{min}^b \quad (10)$$

Values of b different from 1.0 indicated a curvilinear function.

RESULTS

Whole blood/plasma ratio. Relationships between whole blood/plasma ratios and plasma concentrations over the course of infusion and oral dose treatment of two patients are shown in Fig. 1. The maximum ratio when plasma concentrations approach zero show high variability between patients, with values ranging from

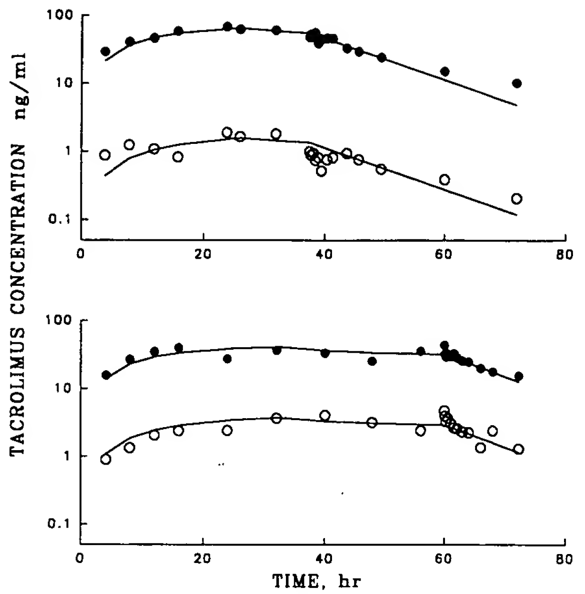

Fig. 2. Time pattern of tacrolimus concentrations in whole blood *(solid circles)* and plasma *(open circles)* during and after the initial courses of drug infusion in two typical patients. *Circles* are experimental data, and *lines* show least-squares fitting of the data to equations 2 and 3.

13 to 114. The calculation of binding parameters B_{max} (418 ± 258 ng/ml erythrocytes) and K_D (3.8 ± 4.7 ng/ml) was complicated by the narrow range of plasma concentrations of tacrolimus. However, good correlation between observed values and those fitted by the model (equation 1) was observed for most patients.

Infusion phase. The time pattern of tacrolimus concentrations in plasma and whole blood over the course of the initial infusions and washout are shown for two of the 16 patients in Fig. 2. The rise of tacrolimus concentrations to steady-state and the subsequent washout phase were generally well characterized by the model equations. The profiles were typically parallel and the whole blood data tended to be less erratic than the plasma concentrations. The computer program worked well in tracking any changes in infusion rate or the cessation of tacrolimus infusion before oral dosing with the drug. The intensity of sample collection and the joint plasma and blood fitting process allowed generation of reasonable pharmacokinetic parameters in spite of occasional deviations in the data.

The average and range of the generated pharmacokinetic parameters are listed for the group of patients in Table II. Fig. 3 shows the array of parameters related to whole blood and plasma tacrolimus concentrations; the $t_{1/2}$ and the maximum whole blood/plasma ratio are parameters common to both plasma and whole blood. The overall variability in the disposition of tacrolimus among the group of 16 liver transplant patients was moderate, with coefficients of variation of 32% to 49% for the various pharmacokinetic parameters. The mean plasma clearance was 124 ± 47 (SD) L/hr or 1.68 ± 0.57 L/hr/kg. The volume of distribution averaged 2223 ± 1119 L or 30.1 ± 14.7 L/kg. This indicates that tacrolimus distributes extensively outside of plasma. The $t_{1/2}$ of the drug averaged 12.1 ± 4.7 hours, with one patient exhibiting an exaggerated value of 22.6 hours. These numerical values are reasonable in view of the fact that tacrolimus nears steady-state concentrations in about 36 hours (Fig. 2). The distribution of tacrolimus into red blood cells is both extensive and variable, producing smaller values of CL_B and V_B than parameters based on plasma concentrations.

It is of interest to observe (Fig. 4) that CL_p shows a strong linear relationship (CL_p = 0.553 + 0.022 blood/plasma ratio; r = 0.65, t = 3.17) with the maximum blood/plasma ratio, whereas CL_B seems to be independent (r = −0.30, t = 2.10). Similar findings occur with V_p (V_p = −0.165 + 0.547 blood/plasma ratio; r = 0.73, t = 4.04) and V_B (r = 0.30, t = 1.18) and maximum blood/plasma ratio as also shown. Thus the blood binding of tacrolimus appears to be a major factor in accounting for interpatient differences in pharmacokinetics.

Oral dose kinetics. Fig. 5 shows the individual whole blood and plasma concentration versus time profiles for three 12-hour oral dose studies for one patient. The experimental data and fitted curves are usually somewhat flat. The one-compartment model with a time lag, first-order absorption, and nonlinear binding with erythrocytes characterized the patient data well.

A summary of the fitted absorption parameters for each patient and study phase is provided in Fig. 6 and Table II. There was good agreement (r^2 = 0.71) between the two methods of generating F (equation 7 versus equations 8 and 9). Overall, the bioavailability of tacrolimus averages 25% ± 10% by fitting. One patient had a high F value of 80% and one had a low value of 4%; all others ranged between 12% and 54%. The time lag was brief, with a mean of 0.39 hour and a range from 0 to 2.0 hours. The value of k_a was variable, with a mean of about 4.0 hr^{-1}; however, the patient data tended to cluster with very high (6 to 8 hr^{-1}) or very low (0 to 2 hr^{-1}) k_a values.

There appeared to be as much interpatient variabil-

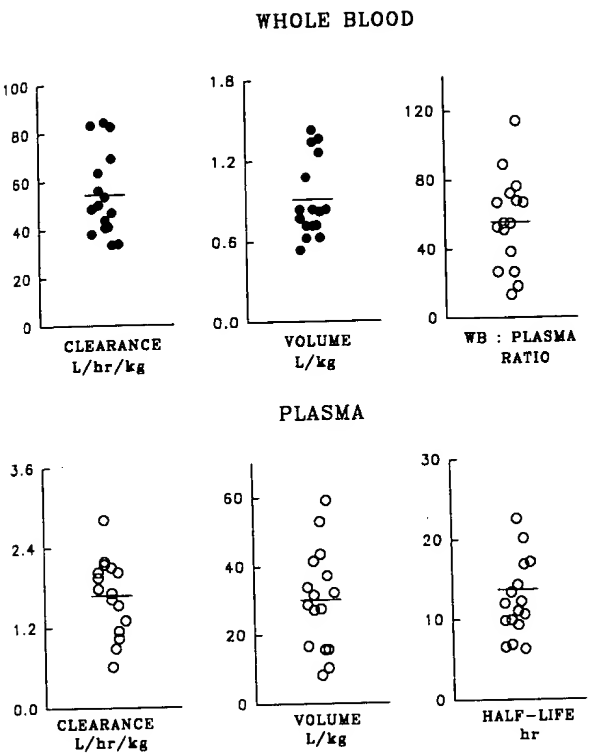

Fig. 3. Range of pharmacokinetic parameters of tacrolimus in 16 liver transplant patients as related to whole blood and plasma drug concentrations.

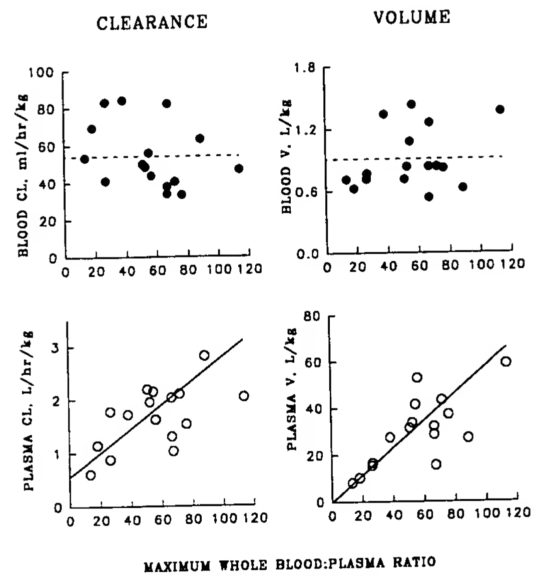

Fig. 4. Relationship between plasma *(open circles)* and whole blood *(solid circles)* clearances (CL) and volumes of distribution (V) of tacrolimus and the whole blood to plasma distribution ratios in 16 liver transplant patients. *Broken lines* represent mean values. Perpendicular least-squares regression analysis produced the *solid lines*.

Table II. Pharmacokinetic parameters for tacrolimus in liver transplant patients

Parameter	*Symbol*	*Mean*	*SD*	*Range*
Plasma				
Clearance (L/hr/kg)	CL_P	1.69	0.57	0.61-2.81
Volume (L/kg)	V_P	30.1	14.7	8.2-59.0
Whole blood				
Clearance (ml/hr/kg)	CL_B	54.1	17.3	33.4-84.0
Volume (L/kg)	V_B	0.906	0.290	0.535-1.430
Common				
Half-life (hr)	$t_{1/2}$	12.1	4.7	6.3-25.3
Maximum blood/plasma ratio	BPR	55.5	26.8	13.4-114
Bioavailability				
Lag time (hr)	t_{lag}	0.39	0.39	0.0-2.0
Absorption rate constant (hr^{-1})	k_a	4.48	2.99	0.14-8.0
Fraction absorbed	F	0.25	0.10	0.04-0.89

ity in F as there was within patients. Fig. 7 was constructed to determine whether there was any relationship of F with the time elapsed since initiation of therapy. The graph indicates a tendency for more variable F values at the time of the first study, but the general consistency of bioavailability with time is evident.

DISCUSSION

Infusion phase. The disposition kinetics of tacrolimus are reasonably consistent among this group of liver transplant patients considering that the data were obtained immediately after the transplant procedure when the patients were in acute recovery and that the drug is essentially completely metabolized by the liver

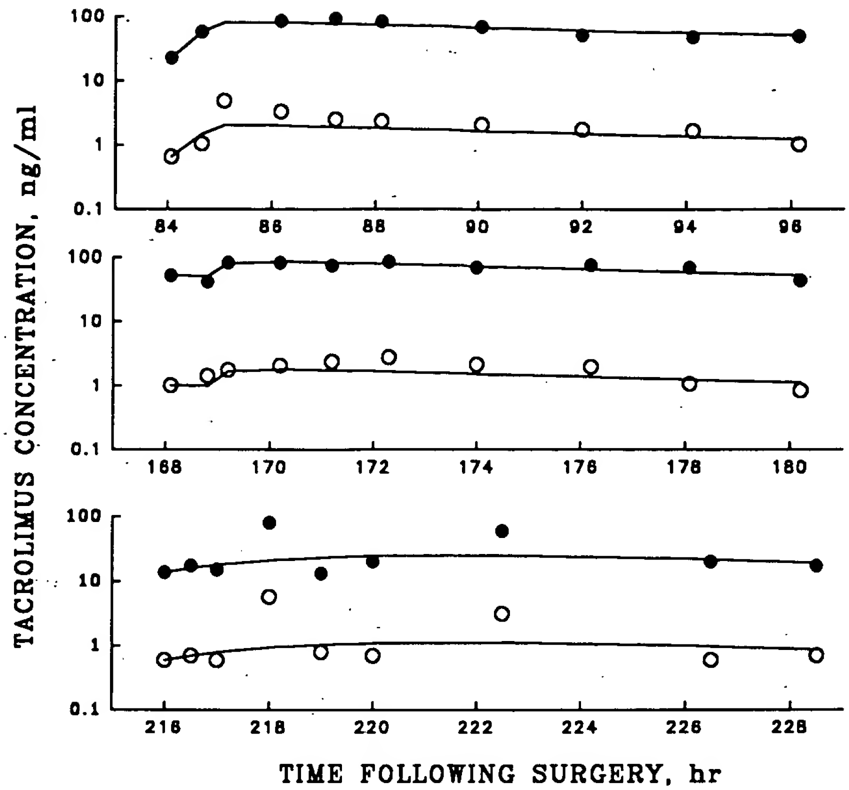

Fig. 5. Time pattern of tacrolimus concentrations in whole blood *(solid circles)* and plasma *(open circles)* after oral doses of drug in one patient at various times following hepatic transplantation. *Circles* are experimental data, and *lines* show least-squares fitting of the data to equation 7.

as we and others[5] have found practically no drug in urine. Also of concern are the variability in the enzyme immunoassay procedure[7] and the partial inclusion of immunoreactive metabolites in the assay.[12] The large number of samples collected offsets occasional errant data.

Venkataraman et al.[5] report pharmacokinetic parameters for tacrolimus in patients that are similar to present values. Because of the large apparent plasma clearance, they classified tacrolimus as a "high clearance" drug. Indeed our CL_p of 1.68 L/hr/kg exceeds the normal hepatic plasma flow of about 0.7 L/hr/kg. However, the distribution of tacrolimus into red blood cells complicates this issue because the blood clearance of 0.0541 L/hr/kg or 54.1 ml/hr/kg is less than the normal hepatic blood flow of about 1200 ml/hr/kg. Some drug diffuses out of red cells during passage through the liver, thus offering a "true" hepatic clearance that falls between the CL_B and CL_p values.[10]

In view of the above dilemma, it is noteworthy that patients with the highest blood/plasma ratio have highest CL_p and V_p values and, conversely, lowest CL_B and V_B parameters (Fig. 4). The clearance data appear to be most readily explained by the possibility that the drug is poorly extracted from whole blood; thus patients who exhibit the strongest red cell binding have the lowest CL_B. This is the traditional pharmacokinetic thinking with regard to the role of plasma protein binding in hepatic extraction of low clearance drugs from plasma. In turn, the plasma clearance values correlating to the blood/plasma ratio would be incidental to the occurrence of blood binding, which simply shifts drug out of plasma into red cells. After hepatic extraction of tacrolimus, the circulating drug would reequilibrate between red cells and plasma and hepatic venous blood would mix with blood in the vena cava, producing the present ratios that are found in peripheral blood. As assessed by ultracentrifugation, tacrolimus binding in plasma is weak, averaging 72% ± 4% in transplant patients, and is thus not an important factor.[13] Perfused organ studies using the rabbit, which has blood binding of tacrolimus similar to humans, verify that red cell binding restricts the apparent clearance of the drug.[10]

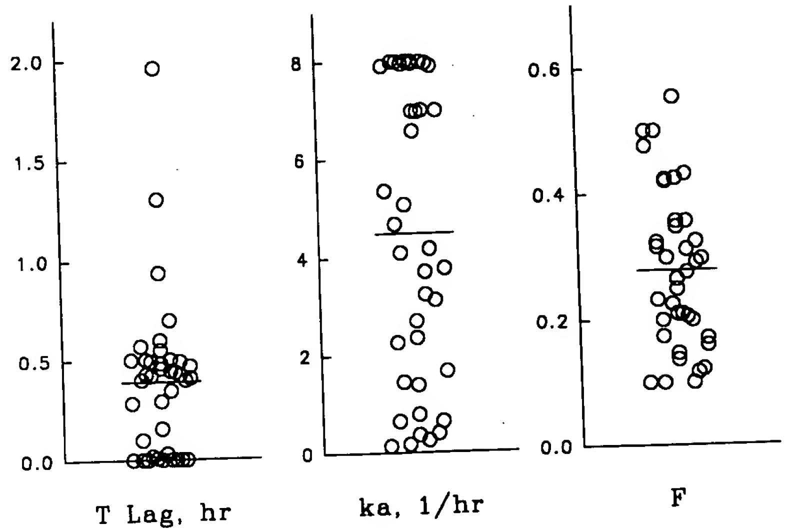

Fig. 6. Summary of tacrolimus absorption parameters for each oral dose study in all liver transplant patients. t_{lag}, Absorption lag time; k_a, absorption rate constant; F, bioavailability.

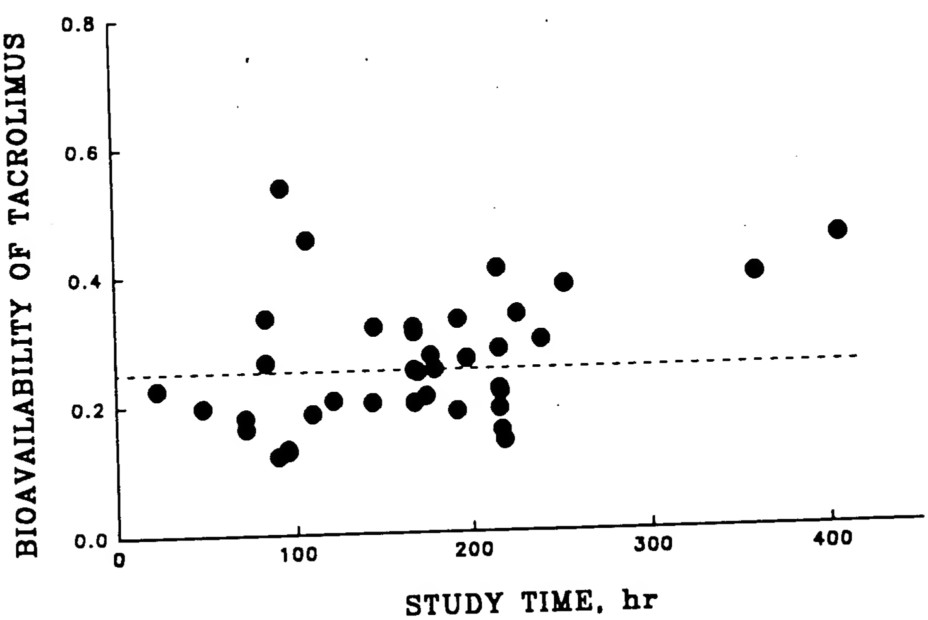

Fig. 7. Bioavailability of tacrolimus in all liver transplant patient studies in relation to the time elapsed since initiation of therapy.

The findings and explanation provided above lend insight into our previous observation of a nonlinear relationship between the blood/plasma ratio and steady-state trough concentrations of tacrolimus in liver transplant patients.[7]

The relationship of volumes of distribution to the blood/plasma ratio of tacrolimus are similar (Fig. 4) to the clearance data. The greater blood cell binding leaves less drug in plasma, producing a larger volume relative to plasma.

Absorption of tacrolimus. The bioavailability of tacrolimus was reasonably consistent among the pa-

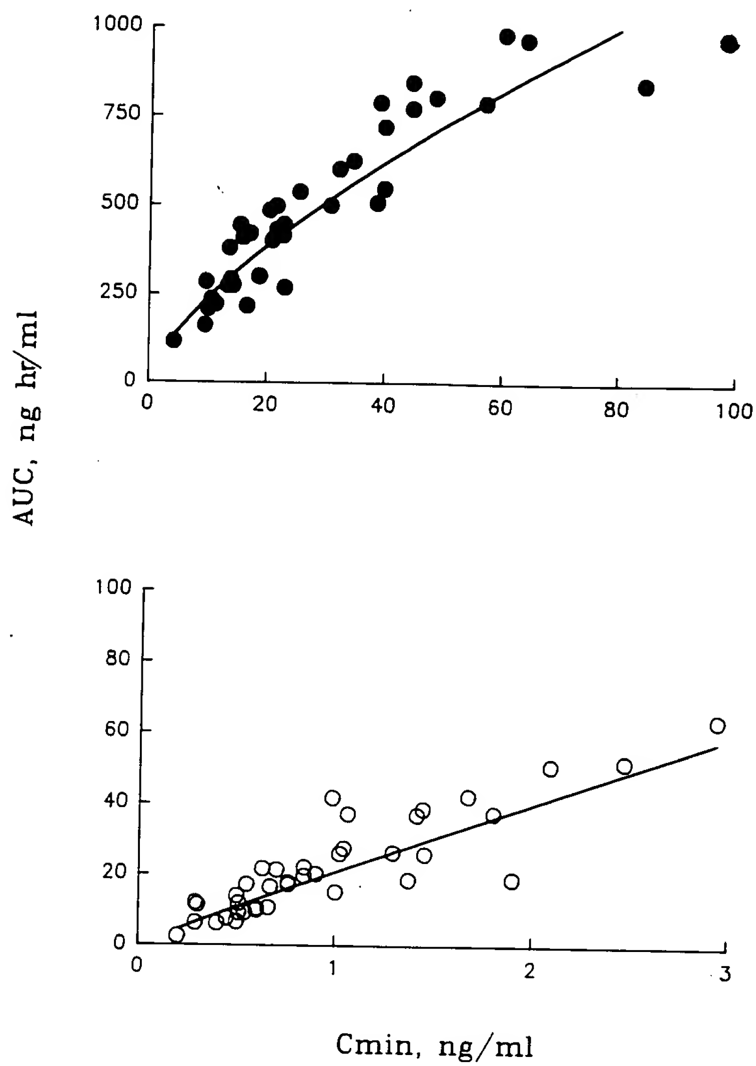

Fig. 8. Relationship between AUC and 12-hour C_{min} values of tacrolimus in whole blood *(solid circles)* and plasma *(open circles)* for each oral dose study phase in all liver transplant patients. AUC, Area under the concentration–time curve; C_{min}, minimum concentration.

tients, doses, and times (Fig. 7), with a mean F of 25% ± 10%. The time patterns were somewhat variable, with patient profiles ranging from flatness to abrupt rises (Fig. 5). This was reflected in the diversity of k_a values (Fig. 6). Nevertheless, a consistent amount of the drug was eventually absorbed, as indicated by the F values. Because tacrolimus is actually a low clearance drug with CL_B best reflecting its elimination, the low F values represent the incompleteness of tacrolimus absorption rather than first-pass loss of the compound. This interpretation is supported by direct studies of the gastrointestinal absorption of tacrolimus in rats (Kobayashi M, personal communication, November 1992) and rabbits.[10]

Trough concentrations. Patient therapy with tacrolimus is accompanied by monitoring trough or 12-hour C_{min} concentrations in plasma and whole blood to seek relationships with efficacy and toxicity.[2,3,8] A common assumption in therapeutic drug monitoring is that the C_{min} will be a reasonable index of overall patient exposure to the drug. Both the plasma and whole blood AUC values are directly proportional to the respective C_{min} concentrations (Fig. 8). There is less variability in the relationship based on whole blood values, but a slight curvilinear pattern suggests that high C_{min} concentrations may exaggerate the relative exposure of patients to tacrolimus. The values (and 95% confidence limits) of the b coefficients in

equation 10 were 0.94 (0.77% to 1.11%) for plasma and 0.69 (0.60% to 0.78%) for whole blood, indicating a meaningful deviation from linearity for only the latter relationship. The fitted values of a were 20.8 for plasma and 48.7 for whole blood. Without the highest two data points, which appear to deviate from the data pattern, the relationship between AUC and C_{min} for whole blood appears to be linear (r^2 = 0.889).

The good relationship between AUC and C_{min} values may be of clinical advantage in therapeutic monitoring. For cyclosporine, AUC values may afford tighter control of patient therapy.[14]

Clinical relevance. Hepatic transplant patients exhibit nonlinear (Fig. 1) and variable (Fig. 3) blood/plasma ratios of tacrolimus, which accounts for much of the variation in clearance and volume (Fig. 4). The extensive uptake of tacrolimus by red cells appears to be caused by a tacrolimus binding protein with properties similar to a specific protein found in lymphocytes and in various tissues.[15] Although blood binding is an important controlling factor for the pharmacokinetics of tacrolimus, it is premature to presume that either plasma or whole blood will be preferable for therapeutic monitoring. For cyclosporine, concentrations in either biological fluid can serve as useful indexes for maintaining allograft function and preventing adverse reactions,[16] but whole blood provides better discrimination and offers conveniences in sample handling.[17] Tacrolimus dosages for patients were originally devised on the basis of the generation of plasma concentrations that were similar to in vitro concentrations that inhibited lymphocyte function,[18] and free drug is usually that which equilibrates with receptor sites. One clinical study argues for plasma monitoring of tacrolimus, with an apparent therapeutic range from 0.4 to 1.2 ng/ml.[8] Ongoing clinical studies that monitor efficacy and toxicity may resolve the issue of the relative merits of plasma versus blood monitoring.

This study shows several features of tacrolimus that rationalizes present dosing regimens. The choice of an infusion is justified on the basis of the rapid attainment of consistent and therapeutic plasma and blood concentrations, which are readily maintained over the first 3 days of therapy. The moderate $t_{1/2}$ of about 12 hours confirms the utility of a twice-daily oral dosing regimen which, in turn, produces only modest fluctuations in C_{max} and C_{min} concentrations (Fig. 5). The C_{min} values are proportional to AUC, which is of benefit for therapeutic monitoring. The present dosing regimens produce blood and plasma concentrations of tacrolimus that provide a reasonable starting point in patient therapy.

Analytic and computational assistance were provided by Ms. Robin D'Ambrosio, Ms. Suzette Mis, and Ms. Nancy Pyszczynski.

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Tacrolimus Analysis: A Comparison of Different Methods and Matrices

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Summary: We determined the trough blood and plasma concentrations of tacrolimus from the day of transplantation through 30 days posttransplantation in four liver and four kidney transplant patients by three different methods. The first method involved a solid phase extraction of the blood or plasma using Sep-Pak columns (SPs) followed by quantitation of tacrolimus using an enzyme-linked immunosorbent assay (ELISA); the second method involved a liquid–liquid extraction using methylene chloride (MC) followed by quantitation of tacrolimus using the ELISA, and the third method involved a high-performance liquid chromatography (HPLC) fractionation of the extract obtained from the solid-phase extraction and quantitation of tacrolimus in the fractions by ELISA. The trough plasma tacrolimus concentrations ranged from 0.1 to 5.2 ng/ml. While the trough plasma concentrations of tacrolimus were similar and independent of the method of analysis in kidney transplant patients and in liver transplant patients with normal biochemical profile, in patients with liver dysfunction, tacrolimus plasma concentrations were higher when measured by SP-ELISA and MC-ELISA methods as compared to the HPLC-ELISA method. In plasma samples obtained from liver transplant patients with liver dysfunction, the presence of some metabolites that cross-reacted with the antibody used in the ELISA could be documented in the HPLC fraction corresponding to the metabolites. This indicates that while tacrolimus metabolites that cross-react significantly with the antibody used in the ELISA do not accumulate in kidney transplant patients, they can appear in the plasma of patients with liver dysfunction. The trough blood tacrolimus concentrations in patients were significantly higher than the corresponding plasma concentrations and ranged from 1.4 to 107 ng/ml. The trough blood tacrolimus concentrations were similar and independent of the method of analysis in kidney and liver transplant patients, suggesting unchanged tacrolimus to be the major component in the blood. The HPLC fractions corresponding to the metabolites of tacrolimus did not contain any components that cross-reacted with the antibody used. This study documents that the methods used in this study for the analysis of blood concentrations of tacrolimus appear to be specific for the parent tacrolimus and can be used in future pharmacokinetic and clinical studies. **Key Words:** Analysis—Immunosuppression—Tacrolimus—Therapeutic monitoring.

Therapeutic monitoring of immunosuppressive drugs is an integral part of optimizing drug therapy in transplant patients because of the narrow therapeutic index of these agents. Since immunosuppressive drugs are used on a long-term basis, trough

Received June 7, 1994; accepted August 22, 1994.
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blood or plasma concentration measurement of immunosuppressive drugs in organ transplant patients will also help in assuring compliance with the drug treatment protocol. Tacrolimus, a recently approved immunosuppressive drug, is effective in preventing graft rejection in patients after liver, kidney, and heart transplantation (1). Large interindividual variability in the pharmacokinetics (2) and the nephrotoxicity of tacrolimus (3) necessitates routine monitoring of this agent in transplant patients. Tacrolimus has been measured in plasma by an enzyme-linked immunosorbent assay (ELISA) that was originally developed by Tamura et al. (4), or by a modification of this ELISA (5–8), or by high-pressure liquid chromatography (HPLC)-ELISA (9,10), or by HPLC after derivatizing with danzyl hydrazine (11), or by a bioassay (12). Blood concentration of tacrolimus has been measured by ELISA (6,7,8,13), by a microparticle enzyme immunoassay developed by Abbott Laboratories (14–16), by a radioreceptor assay (17), or by HPLC-mass spectrometry (18,19).

Recently, we compared the plasma tacrolimus concentrations in kidney and liver transplant patients as measured by ELISA after a solid-phase extraction or a liquid–liquid extraction using methylene chloride or after a solid-phase extraction and HPLC fractionation to separate unchanged tacrolimus from metabolites that might be coextracted (10). We observed significant differences in the plasma concentration of tacrolimus between the different methods used only in samples obtained from patients with impaired liver function as determined by elevated bilirubin concentrations. This study was designed to extend our observations to whole blood tacrolimus concentration measurements in liver and kidney transplant patients during the immediate postoperative period.

MATERIALS AND METHODS

Materials

Tacrolimus pure drug, the monoclonal antibody for tacrolimus, and tacrolimus peroxidase enzyme conjugate were supplied by Fujisawa Pharmaceuticals, Osaka, Japan. Anti-mouse IgG was purchased from Atlantic Antibodies (Stillwater, MN, U.S.A.). Sep-Pak columns (SPs) (C-18) were obtained from Waters (Milford, MA, U.S.A.). O-Phenylenediamine was purchased from Sigma (St. Louis, MO, U.S.A.). Methylene chloride and methanol were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). The HPLC column used, a u-Bondapak C-18 column that is 15 cm long with an internal diameter of 3.9 mm was maintained at 60°C and the column eluents were monitored at 214 nM. Using a mobile phase of 80% methanol and 20% water (acidified to pH 6 with HCl) at a flow rate of 1.5 ml/min, the retention time of tacrolimus was 4.8 min.

Methods

Clinical Protocol

Patients received i.v. tacrolimus (0.1 mg/kg/day) as a continuous i.v. infusion during the immediate postoperative period and for up to 3–7 days after surgery. Oral therapy was initiated as soon as the patients were able to tolerate oral intake. Tacrolimus (0.1–0.24 mg/kg/day) was administered orally twice daily. Multiple serial blood samples were collected in heparinized tubes prior to the morning dose of tacrolimus from four liver and four kidney transplant patients from day 1 after surgery until approximately 30 days posttransplantation.

Analysis

Aliquots of the whole blood samples were separated and maintained at 4°C until analysis. The rest of the blood samples were incubated at 37°C for 45 min and centrifuged at 37°C for 10 min and the plasma obtained was analyzed on the same day or frozen at −20°C until analysis. The calibration curve for whole blood consisted of blank blood samples spiked with tacrolimus concentrations of 0, 0.8, 4, 8, 16, 40, and 64 ng/ml. Blood and plasma samples were extracted by the solid-phase extraction method, or by the liquid–liquid extraction method using methylene chloride, as described previously (10), except for the use of 1.0 N of HCl to assure complete lysis of whole blood samples prior to extraction. Additional samples were also extracted by the solid-phase extraction method and subjected to HPLC separation (as described in reference 10), and the fractions corresponding to the intact tacrolimus (fraction 2, 3.6–6 min) and tacrolimus-related materials (fraction 1, 0–3.6 min) were separately collected and evaporated to dryness under nitrogen. All the extracts were analyzed by ELISA, with overnight incubation.

A minimum of nine blood and plasma samples was analyzed from each of the patients observed.

Biochemical parameters indicative of the kidney function (serum creatinine level) and liver function (bilirubin and aspartate and alanine aminotransferase levels) were also measured in these patients over the entire study period.

RESULTS

The biochemical profiles of the patients observed are listed in Table 1. While all but one kidney transplant patient had normal liver function test results, not all the liver transplant patients had normal kidney function throughout the entire course of study. The interday coefficients of variation of the three methods used varied from 4.2 to 19.7% (Table 2), and were similar between the three methods used.

Figures 1 and 2 illustrate tacrolimus blood concentrations as measured by the three methods in liver and kidney transplant patients, respectively. In all the patients, the whole blood concentration of tacrolimus was very similar as measured by ELISA after the three different extraction/separation procedures. The regression equations of the tacrolimus concentrations measured by HPLC-ELISA versus SP-ELISA or MC-ELISA in whole blood are listed in Table 3. There was a significant correlation (0.821–0.949) between the two variables evaluated, and the slope of the lines was near unity (0.92–1.1).

Figures 3 and 4 illustrate the plasma concentrations of tacrolimus in liver and kidney transplant patients as measured by the three methods. Tacrolimus plasma concentrations were similar as measured by the three methods in the kidney transplant patients at all times. However, in the liver transplant patients, when the serum bilirubin concentrations were elevated, the tacrolimus concentrations measured by SP-ELISA and MC-ELISA were higher than the values obtained by the HPLC-ELISA method. As the bilirubin concentrations recovered toward normal values, the concentration of tacrolimus measured by all three methods was almost identical. Correspondingly, Table 3 shows a slope of greater than unity (1.87 and 1.75, respectively) when plasma tacrolimus concentrations measured by SP-ELISA or MC-ELISA were compared with HPLC-ELISA measurements.

Figure 5 illustrates the correlation between plasma concentrations and whole blood concentrations in liver and kidney transplant patients as measured by all three methods. Blood and plasma con-

TABLE 2. *Between-run precision of different assay methods*

	Whole blood			Plasma		
	Mean, ng/ml	% CV	No.	Mean, ng/ml	% CV	No.
Solid-phase extraction						
LC	13.2	18.2	7	0.7	14.2	18
MedC	23.4	19.7	7	2.2	13.6	18
HC	36.3	15.4	7	5.8	12.0	18
Methylene chloride extraction						
LC	11.1	5.4	7	0.6	16.6	8
MedC	18.5	8.0	7	1.8	11.1	6
HC	30.6	13.0	7	5.7	15.7	5
HPLC						
LC	15.5	13.5	3	0.7	14.3	7
MedC	28.9	4.2	3	2.3	17.4	7
HC	35.1	5.4	4	5.6	10.7	7

CV, coefficient of variation; LC, low control; MedC, medium control; HC, high control; HPLC, high-performance liquid chromatography.

TABLE 1. *Biochemical profile in four liver and four kidney transplant patients*

	Creatinine, mg/dl		Bilirubin, mg/dl		AST, IU/L		ALT, IU/L	
Patients	Immediate postoperative period	3 wk after transplant	Immediate postoperative period	3 wk after transplant	Immediate postoperative period	3 wk after transplant	Immediate postoperative period	3 wk after transplant
Kidney								
CK	3.6	1.7	0.4	0.3	46	20	38	66
KW	1.2	1.2	0.9	0.7	712	33	1,476	89
JG	0.8	2.9	0.5	0.8	46	18	34	42
PC	2.3	2.2	0.7	0.6	20	44	27	140
Liver								
RC	4.1	3.3	8.0	2.9	101	90	93	96
GB	2.7	4.4	8.5	1.6	6,605	24	996	31
IZ	2.4	1.4	13.4	14.3	1,247	91	644	127
LT	1.6	1.3	14.5	26.3	863	136	725	495

AST, aspartate aminotransferase; ALT, alanine aminotransferase.

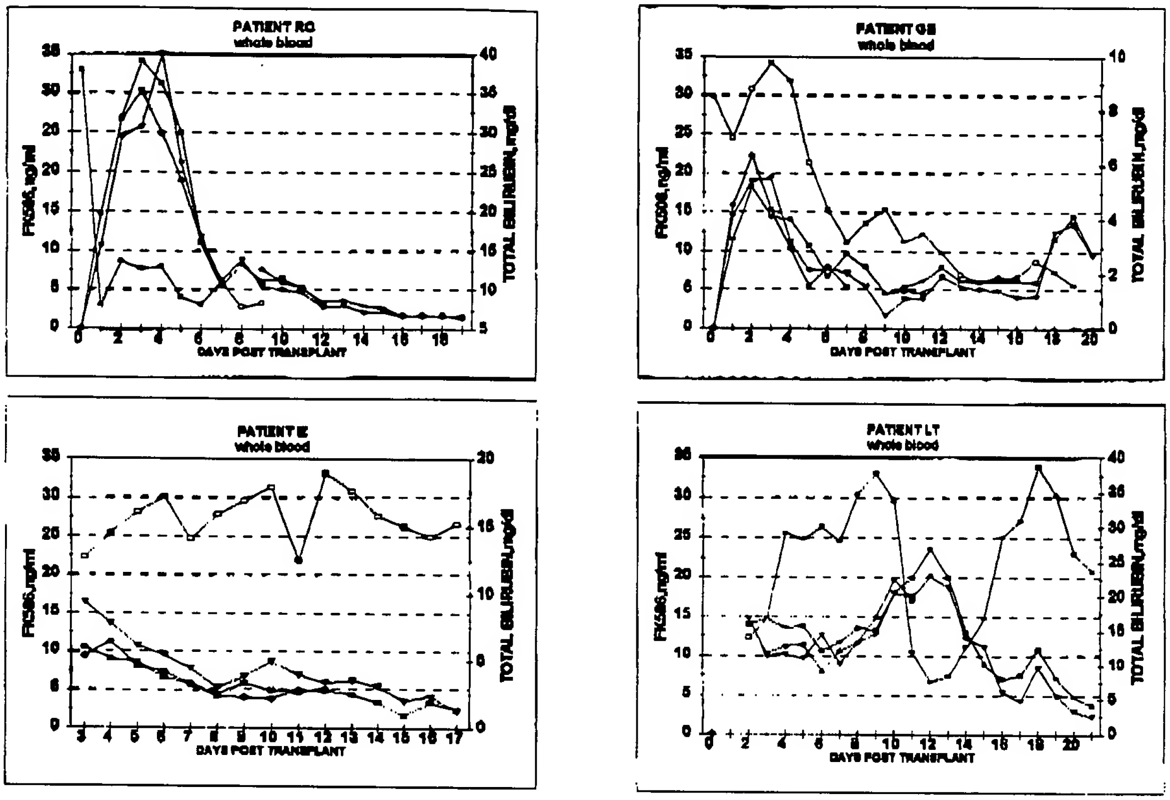

FIG. 1. Whole-blood FK 506 concentrations as measured by Sep-Pak enzyme-linked immunosorbent assay (ELISA) (triangles), methylene chloride ELISA (black boxes), high-performance liquid chromatography ELISA (circles), and bilirubin (white boxes) in four liver transplant patients.

centrations were poorly correlated ($r \leq 0.59$) with each other. The overall slope of blood versus plasma concentrations (4.1) in liver transplant patients was lower than the corresponding overall slope (11.7) in the kidney transplant patients (Table 4). The slope of the line comparing the plasma HPLC-ELISA concentrations versus whole blood HPLC-ELISA concentrations in liver transplant patients was also lower than the corresponding slope in the kidney transplant patients. The tacrolimus equivalent in fraction 1 (corresponding to HPLC fraction collected from 0 to 3.6 min) was <0.8 ng/ml in all cases.

DISCUSSION

In this study we compared three methods of measuring tacrolimus concentrations in plasma and whole blood. The sensitivity and the interday variations of the three methods were comparable and were acceptable for routine clinical use.

Over the past few years we have been using the SP extraction procedure for measuring plasma concentrations of tacrolimus in transplant patients. In a recent publication (10) we reported our observation of identical tacrolimus concentrations in plasma as measured by SP-ELISA, MC-ELISA, and HPLC-ELISA in kidney transplant patients and in liver transplant patients with biochemical profiles indicative of normal liver function. In patients with abnormal liver function, as determined by serum bilirubin concentrations >2 mg/dl, the SP-ELISA method tends to give higher estimates of tacrolimus concentration in comparison to the MC-ELISA method. However, both of these methods give values that are higher [slope of SP-ELISA vs. HPLC-ELISA was 1.53 (n = 40), vs. slope of MC-ELISA vs. HPLC-ELISA, 1.13 (n = 40)] than the HPLC-ELISA method, which measures only the parent tacrolimus in plasma. This is believed to be the result of coelution of some tacrolimus metabolites in the SP and MC extraction that cross-react with the antibody used in the ELISA procedure. When data from patients with a serum bilirubin concentration of <2 mg/dl were analyzed, the slope of the SP-ELISA versus HPLC-ELISA was 1.19 (n = 27),

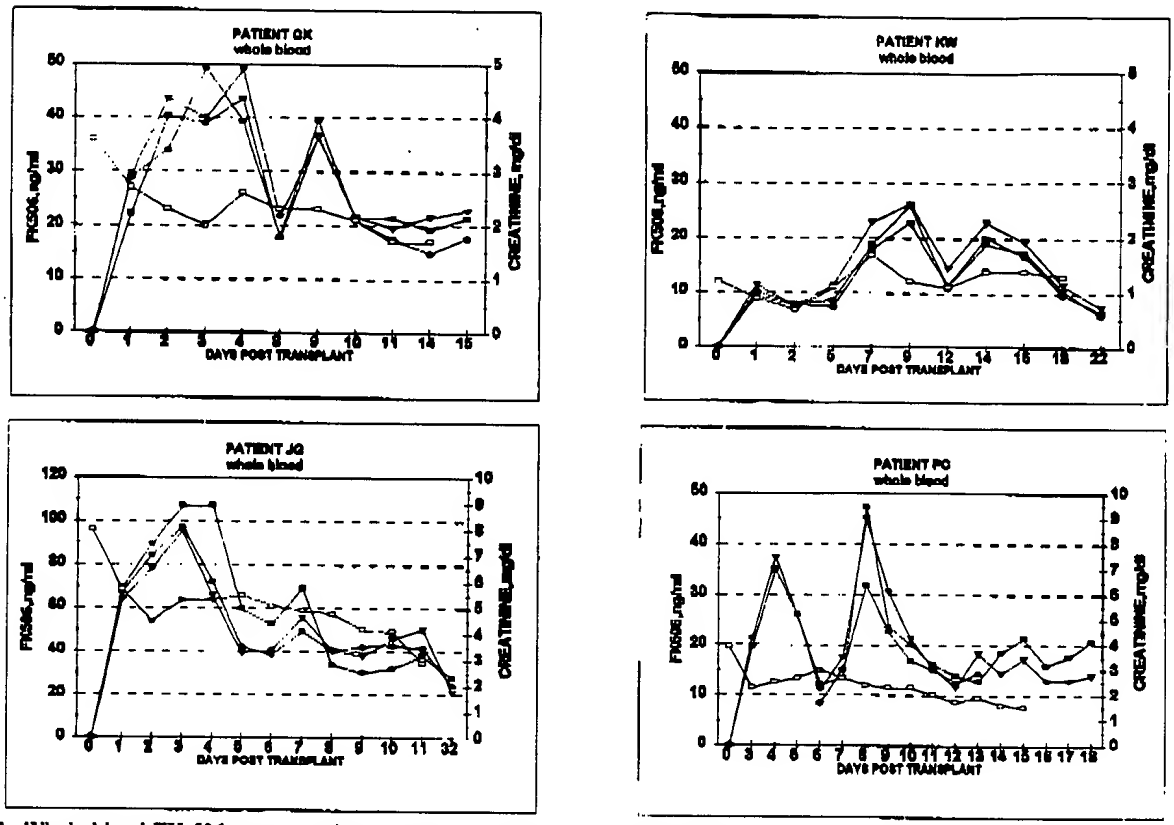

FIG. 2. Whole-blood FK 506 concentrations as measured by Sep-Pak enzyme-linked immunosorbent assay (ELISA) (triangles), methylene chloride ELISA (black boxes), high-performance liquid chromatography ELISA (circles), and creatinine (white boxes) in four kidney transplant patients.

and the slope of the MC-ELISA vs. HPLC-ELISA was 1.07 (n = 27).

Our current observations of tacrolimus concentrations in plasma obtained from liver and kidney transplant patients are in good agreement with the published data (5,9,10) but differ in the magnitude of difference between the SP-ELISA and the MC-ELISA (5,9). Our results are not in agreement with those of a recent report (15) documenting poor correlation (r = 0.41) between the MC-ELISA and the SP-ELISA of plasma. This may be due to the differences in the temperature used to separate the plasma from whole blood (37°C in this study, vs. 24°C in the report cited earlier), the high coefficient of variation at low concentrations measured in the published study, and minor differences in the actual extraction procedures used. The relationship between SP-ELISA and the MC-ELISA reported by Winkler et al. (15) is also different from the original report by Kobayashi et al. (5).

In this study, we observed similar whole blood concentrations of tacrolimus as measured by all three methods, as indicated by slopes of 0.92–1.1 and correlation coefficients of 0.82–0.95 (when SP-ELISA and MC-ELISA were compared to HPLC-ELISA), independent of the functional status of the liver. This would suggest that the parent tacrolimus

TABLE 3. *Correlation analysis of different methods with reference to HPLC method*

	Method	Slope	Intercept	r^2	No.
Whole blood					
liver transplant	MC ELISA	1.0	0.8	0.90	40
	SP ELISA	0.9	1.5	0.85	40
kidney transplant	MC ELISA	1.1	−1.8	0.82	38
	SP ELISA	0.9	3.7	0.95	45
Plasma					
liver transplant	MC ELISA	1.8	0.0	0.85	39
	SP ELISA	1.9	0.1	0.89	39
kidney transplant	MC ELISA	0.9	0.3	0.92	44
	SP ELISA	1.0	0.3	0.82	44

HPLC, high-performance liquid chromatography; MC, methylene chloride; ELISA, enzyme-linked immunosorbent assay; SP, Sep-Pak columns.

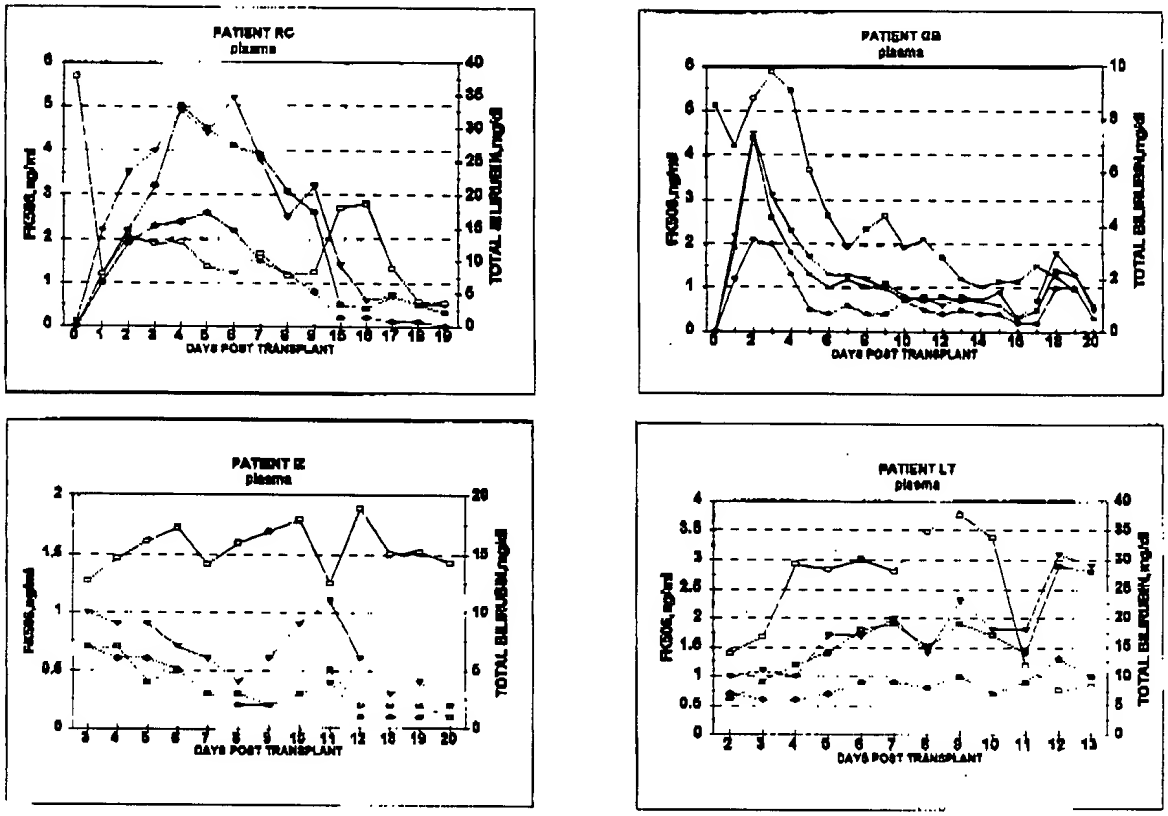

FIG. 3. Plasma FK 506 concentrations as measured by Sep-Pak enzyme-linked immunosorbent assay (ELISA) (triangles), methylene chloride ELISA (black boxes), high-performance liquid chromatography ELISA (circles) and bilirubin (white boxes) in four liver transplant patients.

is the predominant species in the whole blood and that relatively few metabolites accumulate in the blood. While similar correlation (0.786) has been reported for blood tacrolimus concentrations measured by SP-ELISA and MC-ELISA, lower slopes indicative of actual differences between these measurements have been reported (5,15). Our observations with regard to tacrolimus are consistent with the fact that little or no tacrolimus equivalent could be measured in fraction 1, collected on the HPLC. Whether the lack of tacrolimus equivalents in fraction 1 is due to the absence of cross-reacting tacrolimus metabolites in this fraction or to the potential instability of any tacrolimus metabolites in this fraction during the experimental procedure cannot now be clarified.

We have also noted the blood concentration of tacrolimus to be consistently higher [overall mean ± SD blood-to-plasma ratio (B/P) was 12.8 ± 8.4; for kidney transplant patients, B/P was 16.8 ± 9.9; for liver transplant patients, B/P was 9.8 ± 5.5] than the corresponding plasma concentrations in all the patients observed at all times, as has been reported earlier (2,6–8,10,13,15). These observations along with our earlier report would suggest that the parent tacrolimus is the primary species in the red blood cell and that tacrolimus metabolites do not appreciably partition into the red blood cells. The actual concentration of the metabolites that tend to accumulate in the plasma of patients with liver dysfunction appears to be very small compared to the total concentration of tacrolimus in the whole blood. Our conclusions differ from those of the recent publication (15), which suggests that the whole blood SP-ELISA and MC-ELISA are nonspecific and simultaneously measure tacrolimus metabolites as well. While these investigators showed some (10–15%) cross-reactivity of one of the metabolites (dimethyl hydroxy tacrolimus), three other metabolites tested did not show any appreciable cross-reactivity with the monoclonal antibody used in the ELISA procedure. These investigators also interpreted increased tacrolimus concentrations during episodes of liver dysfunction to be due to accumulation of tacrolimus metabolites. It should be realized that increased tacrolimus concentrations dur-

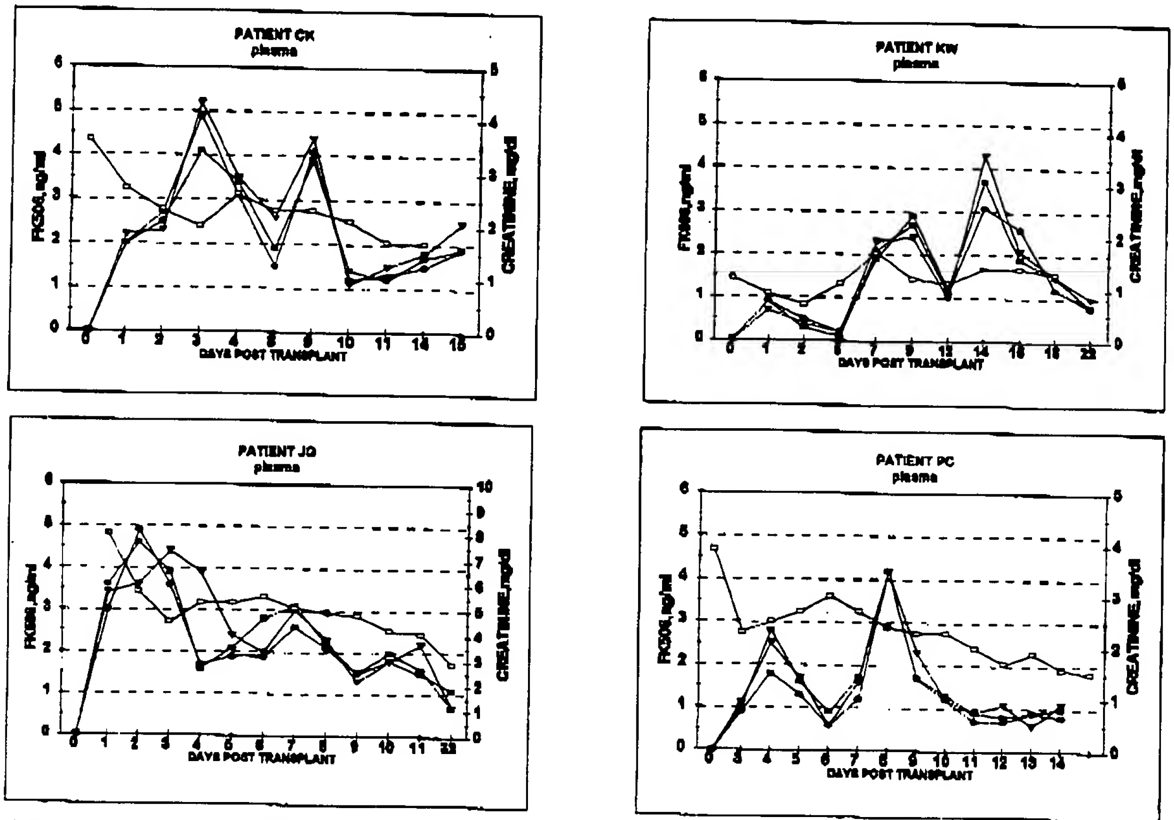

FIG. 4. Plasma FK 506 concentrations as measured by Sep-Pak enzyme-linked immunosorbent assay (ELISA) (triangles), methylene chloride ELISA (black boxes), high-performance liquid chromatography ELISA (circles), and creatinine (white boxes) in four kidney transplant patients.

ing episodes of rejection could very well be the result of impaired tacrolimus metabolism and accumulation of unchanged tacrolimus.

We have observed an overall poor correlation (r = 0.44 in liver and 0.4 in kidney transplant patients, respectively) between the trough blood and trough plasma concentrations. This is consistent with published data (9,15). It is also of interest to note that the slopes of the lines describing the whole blood-to-plasma concentration of tacrolimus were 4.1 and 11.7, respectively, in liver and kidney transplant patients (Fig. 5). When only HPLC-ELISA data were

FIG. 5. Correlation between whole blood and plasma as measured by solid phase extraction, liquid extraction, and high-performance liquid chromatography (HPLC) (circles), in four kidney transplant patients. Correlations between whole blood and plasma as measured by solid phase extraction and liquid extraction (boxes) and by HPLC (triangles) in four liver transplant patients.

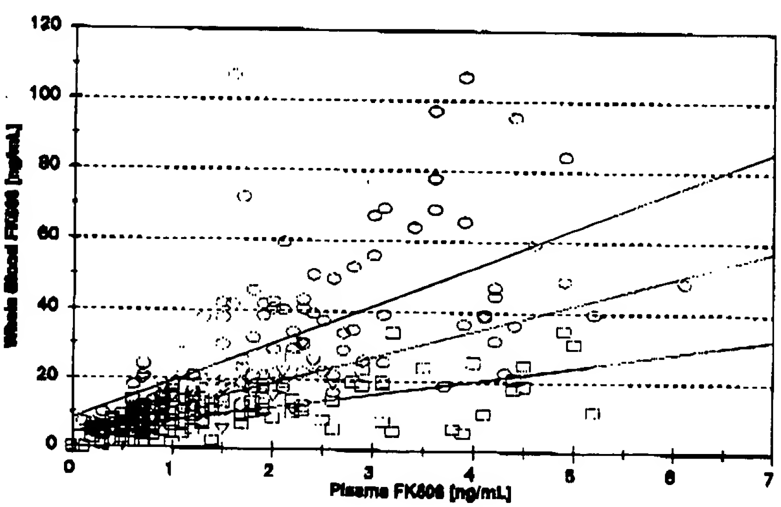

TABLE 4. *FK506—whole blood vs. plasma correlation analysis as measured by different methods*

	Slope	Intercept	r^2	No.
Liver transplant				
Sep-pak ELISA	4.0	3.5	0.52	58
methylene chloride ELISA	4.1	4.7	0.47	58
HPLC ELISA	7.7	3.9	0.59	36
overall (all three methods)	4.1	4.9	0.44	152
Kidney transplant				
Sep-pak ELISA	11.3	7.0	0.50	43
methylene chloride ELISA	12.2	7.5	0.31	37
HPLC ELISA	11.9	7.4	0.44	38
overall (all three methods)	11.7	7.4	0.4	118

ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography.

compared, the slopes were 7.7 and 11.9, respectively, in liver and kidney transplant patients. Our observations in kidney transplant patients indicate that the metabolites of tacrolimus do not accumulate in plasma and/or that they are removed by the extraction process used.

Our observations in the liver transplant patients can be partly explained by the accumulation and simultaneous quantitation of some ELISA cross-reactive metabolites of tacrolimus by SP-ELISA and MC-ELISA in plasma obtained from liver transplant patients with poor liver function. The higher slope of the blood-to-plasma concentration comparison, 11.7 and 7.7, respectively, in kidney and liver transplant patients suggests higher uptake/binding of tacrolimus to the red blood cells or decreased plasma protein binding of tacrolimus in kidney transplant patients as compared to liver transplant patients.

The significant positive intercept (3.5 to 7.5) observed is similar to what has been observed earlier (6,8,15) and is consistent with the reported nonlinear uptake of tacrolimus by the red blood cells (6,8). These observations indicate that it is inappropriate to extrapolate blood tacrolimus concentrations based on plasma tacrolimus concentration measurements made in transplant patients.

Our current observations are also consistent with our previous report that the concentrations of tacrolimus as measured by the three methods are also independent of the route of administration of tacrolimus (10). Tacrolimus is known to be metabolized by the cytochrome P-450 enzyme system (20–22), and this enzyme system is known to be present in the gut. In vitro studies with gut cytochrome P-450 (unpublished observations) provide indirect evidence of the involvement of gut metabolism in tacrolimus disposition. It is possible that in this patient population there was no route-dependent metabolism of tacrolimus, or that the metabolites produced in the gut do not cross-react with the monoclonal antibody used in this assay, or metabolites formed in the gut are removed during the extraction process.

Our study indicates that tacrolimus can be measured in whole blood by ELISA after SP or methylene chloride extraction. The trough blood concentrations of tacrolimus are seven to 12 times as high as the corresponding plasma concentrations. The blood-to-plasma ratio of tacrolimus is dependent on the assay method used, the nature of the organ transplanted, and the functional status of the liver.

Acknowledgment: We acknowledge the financial support of the Pathology Education & Research Foundation (PERF), University of Pittsburgh, and the Veterans Administrative Hospital for this project.

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Validation and Quality Assurance Program for Monitoring Tacrolimus (FK 506) Concentrations in Plasma and Whole Blood

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Summary: Tacrolimus (FK 506), an investigational immunosuppressant drug, is undergoing several trials for various transplantations where protocols call for monitoring plasma and/or whole blood 12-h trough concentrations. Initially, the FK 506 Central Laboratory (FCL) adapted an established enzyme immunoassay (EIA) for FK 506 in plasma and provided clinical monitoring services for hepatic transplantation trials. Within 1 year, 16 clinical sites participating in these trials began direct use of the immunoassay for plasma and whole blood. A five step validation sequence facilitated rapid training and implementation of proficient assay services. All laboratories utilized common reagents, standards, and procedures. Participation in a quality assurance program involved monthly analysis of the three proficiency unknowns supplied by the FCL and reciprocal analysis of five patient samples (cross-checks) by the FCL. The quality of the data produced was assessed by proficiency scores, bivariate regression analysis, and correlations that demonstrated the concordance of their assay results for FK 506 in plasma and whole blood. **Key Words:** Tacrolimus—FK 506—Therapeutic monitoring—Enzyme immunoassay—Liver transplantation—Quality control.

Tacrolimus (FK 506) is an investigational immunosuppressant agent that is being used in clinical trials for hepatic, renal, and other types of transplantations. The drug is 50–100 times more potent than cyclosporine, and the low doses employed produce plasma concentrations that necessitate measurement by immunoassay. Present clinical protocols call for monitoring 12-h trough FK 506 concentrations in all patients, with therapeutic plasma (PL) concentrations and whole blood (WB) concentrations expected to be in the ranges of 0.5–2 ng/ml and 5–20 ng/ml, respectively.

Received February 3, 1993; accepted May 10, 1993.

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Enzyme immunoassay (EIA) for FK 506 in plasma was developed by Tamura et al. (1). Cadoff et al. (2) implemented the assay with a C18 Sep-Pak extraction step; characteristics of this assay method were reported by Warty et al. (3). Jusko et al. (4) validated and employed a methylene chloride extraction procedure, which facilitated both plasma and whole blood analyses of FK 506.

The FK 506 Central Laboratory (FCL) was established at the State University of New York at Buffalo in May 1990. Initially, its purpose was to adapt and validate the EIA for tacrolimus in plasma and whole blood and provide analytical services to all clinical centers outside of Pittsburgh that were utilizing FK 506 in liver transplantation patients. In March 1991, other clinical laboratories began to establish on-site monitoring facilities. During the fol-

lowing months, the FCL provided technical support and training for 16 clinical sites, validating their use of the FK 506 EIA. A five-step validation program was established to assure that all laboratories were operating the FK 506 EIA accurately and precisely prior to implementing routine patient monitoring. Subsequently, all sites participated in a national quality assurance program set up by the FCL and Fujisawa to maintain integrity of results.

This report summarizes the accumulation of validation and quality assurance data for the FK506 EIA as performed with plasma and whole blood samples. Comparability of data produced from several clinical sites was evaluated in relation to results from one reference laboratory (FCL).

METHODS

Assay

FK 506 was extracted from plasma (0.3 ml) or whole blood (0.02 ml) specimens into methylene chloride. The evaporated and reconstituted sample was assayed for FK 506 using a double antibody EIA method. Polyclonal goat-antimouse antibody (IncStar) was precoated onto EIA certified plates (ICN Flow) overnight at 4°C. FK 506 from the sample and FK 506-peroxidase enzyme conjugate competed for sites on the monoclonal anti-FK 506 mouse antibody, which formed an antibody complex with the polyclonal antibody coating the plate wells. Nonspecific sites were blocked using 1% bovine serum albumin in 0.05% Tween 20/phosphate buffer. After washing the unbound substances free, freshly prepared *O*-phenylenediamine HCl solution was added to each well. The reaction was allowed to proceed in the dark and, after 15 min, was terminated by adding 4*N* H_2SO_4 to each well. Color was read at 490 nm. A four-parameter logistic function fit the standard curve and results were calculated using the KinetiCalc 2.026 (Fisher Scientific) software program.

As previously reported (4), the routine assay yielded a lower limit of quantitation (LOQ) of FK 506 of 0.2 ng/ml in plasma and 1.0 ng/ml of whole blood. The higher limit of quantitation (HOQ) exceeded, but was taken as the highest standard for each curve, 120 ng/ml in whole blood and 10 ng/ml in plasma. A conservative approach allowed participating sites to report a range of only 2–80 ng/ml for whole blood and 0.2–5 ng/ml for plasma samples, unless upper range concentrations were appropriately diluted. The coefficient of variation (CV) for quality control (QC) samples in plasma and whole blood typically ranged from 4 to 27% and 4 to 17%, respectively.

Guidelines for assay acceptability were established and maintained at each laboratory (5). Up to six individual absorbances or three entire standard concentrations of the 10 whole blood or 11 plasma standards could be omitted if their predicted concentrations exceeded 30% of nominal values. Six plasma controls were assayed on each plate: two low (0.3 ng/ml), two medium (1.2 ng/ml), and two high (3.0 ng/ml). Four of these six controls, one available at each concentration, had to be within ±30% to accept results from the assay. Controls for whole blood were 4, 15, and 60 ng/ml.

All clinical laboratories followed standard operating procedures identical to those of the FCL. Rigorous documentation was required and site inspections performed to assure that procedural differences between laboratories were minimal.

FK 506 Standards, QCs, and Primary EIA Reagents

Standards and QC preparations were supplied by the FCL for all whole blood assays and nearly all plasma assays. If plasma standards were prepared at clinical laboratories, these were tested against FCL standards to verify accuracy. Primary reagents, such as polyclonal antibody (IncStar), monoclonal anti-FK 506 antibody (Fujisawa Ltd), and FK 506 peroxidase enzyme conjugate (Fujisawa Ltd) were specified and no substitutions allowed.

FK506 methanol stocks were prepared at various concentrations. Ten mg of FK 506 powder (Fujisawa Ltd) were weighed and dissolved in 10 ml of methanol (Burdick and Jackson). Dilutions were prepared in methanol and stored at ≤ -20°C in glass vials with teflon-lined caps for no >3 months. Plasma standards (11 concentrations) and controls (three concentrations) were prepared in pooled citrate-phosphate-dextrose plasma (American Red Cross) by spiking specified volumes of these methanolic stocks (methanol content <1% total volume). Whole blood standards (10 concentrations) and controls (three concentrations) were prepared similarly in heparinized whole blood collected from normal, drug-free volunteers or in purchased EDTA whole blood (ABT Laboratories). Aliquots of standards and controls were transferred to 1.5 ml (0.4 ml

plasma standard or 0.7 ml plasma control) or 0.5 ml (0.1 ml whole blood) polypropylene centrifuge tubes (Laboratory Product Sales) and stored in sealed plastic bags at ≤ −20°C. Standards and controls were shipped to laboratories in dry ice to maintain frozen storage; new batches were supplied every 6 months for plasma and every 4 months for whole blood. Adequate stability (<10% loss) was confirmed to be at least 9 months for plasma and 6 months for whole blood.

VALIDATION PROGRAM

Design

The validation program was designed to teach the FK 506 EIA simply and quickly, allow for easy troubleshooting of problems, and establish rapid quality on-site monitoring to accumulate site-specific data on intra- and interassay variability. The five-step validation program is outlined in Fig. 1 and progressed as follows.

I. FK 506 EIA Variability at Zero and Nonspecific and Intermediate Concentration Measurements

The analyst performed the FK 506 EIA, measuring results for one sample repeated over an entire assay plate. One EIA plate contained only the sample reconstituting buffer (zero FK 506 level). Another EIA plate contained the same, but anti-FK 506 monoclonal antibody was absent (nonspecific level). The last plate contained 0.1 ng/ml of FK 506 in sample-reconstituting buffer. Results generated from each plate were examined overall and with respect to each row, column mean, standard deviation, and %CV. This first step allowed for a quick

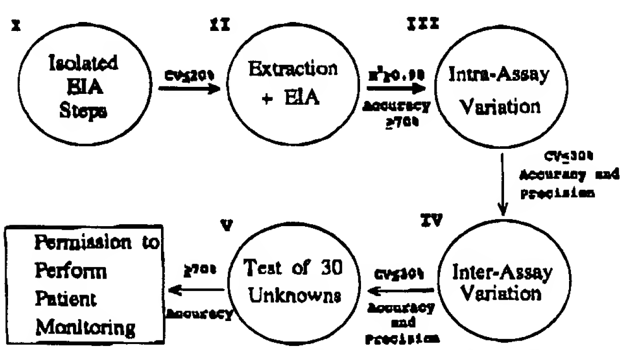


FIG. 1. Schematic for the validation program for FK 506 EIA.

survey of technical pipetting performance as well as reagent and equipment functioning for the EIA portion of the assay. Acceptable results were generally a CV of ≤20% for columns, rows, and mean absorbances for the entire plate. Absorbances from zero FK 506 concentrations were expected to be ≥1.0; nonspecific absorbances, ≤0.1; and 0.1 ng/ml FK 506 absorbances, >0.1 at 490 nm.

II. FK 506 Standard Curve

The analyst extracted samples for one standard curve and measured results via the FK 506 EIA. Results of the standard curve were assessed for goodness of fit, expecting fitted values with ≤30% error from the nominal value within the reportable FK 506 range. Also, duplicate absorbances were evaluated for reproducibility. This step allowed for an initial survey of reagent functioning and equipment performance for the extraction portion of the assay.

III. Intraassay Variability

The analyst extracted samples for one FK 506 standard curve and six replicates of each control level. Mean, standard deviation, %CV, and % true error ([(mean-nominal value)/nominal value] × 100%) were generated for each set of controls. True errors and %CV <30% were considered acceptable.

IV. FK 506 Interassay Variability

The analyst assayed samples for one standard curve and two controls at each level for 5 days. Mean, standard deviation, %CV, and % true error were generated. True errors and %CVs <30% were considered acceptable.

V. FK 506 Blinded Samples

The analyst assayed each of 30 unknown samples in duplicate for FK 506 content. Results were compared to data from the FCL, where each unknown was assayed three or more times. If ≥70% of values were ±30% of the FCL mean value, the validation step was considered acceptable and this part of the program was completed.

NATIONAL QUALITY ASSURANCE PROGRAM

Design

Two types of assessments were utilized to measure interlaboratory variability and performance for FK 506 monitoring (6,7): (a) monthly proficiency samples—three samples spiked with FK 506 were sent monthly to laboratories for assay—and (b) monthly cross-checks—five site-assayed patient samples were shipped from laboratories to the FCL for reanalysis. Figure 2 depicts the overall plan.

Proficiency Samples

Just prior to the beginning of each month, the FCL prepared three batches of spiked FK 506 pools, both in whole blood and plasma. Batches were placed in labeled polypropylene test tubes and stored at $\leq -20°C$ in sealed plastic bags. The FCL analyzed each batch in duplicate over ≥ 3 days. The mean value generated for each batch was considered the target value. Then, at the beginning of each month, the three proficiency samples were shipped frozen in dry ice to each laboratory for analysis. Results were recorded on specific worksheets and faxed from participating sites to the FCL for comparison to the FCL target value; evaluations were faxed back to the sites with approval and acceptance ranges recorded. Those sites with unacceptable results were contacted in order to assess possible problems and for reanalysis of samples.

In October 1991, all three proficiency samples were identical and served as postvalidation measures of intraassay variability. Also, the same sample was sent as the no. 1 proficiency sample for November 1991 and December 1991; these results were accumulated for calculation of post-validation interassay variability. Lastly, blank samples and samples measuring greater than the HOQ were also sent to assure that limits of the assay were measured appropriately.

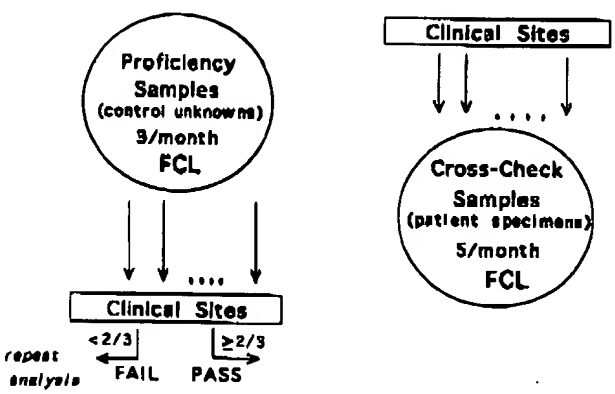


FIG. 2. Schematic for the national quality assurance program for FK 506 EIA.

Monthly Cross-Checks

Each participating laboratory monitoring FK 506 patient samples shipped five patient samples (cross-checks) for reanalysis at the FCL at the end of each month. The FCL would analyze each of these specimens and compare results to site values. If results were significantly different (>30%), the FCL analyzed the samples a second time for confirmation or rejection of the first result.

STATISTICS

Validation, proficiency, and cross-check samples were treated as separate quality assurance measures. For overall comparison of individual samples and laboratories, site values, y, were regressed against FCL values, x, using the modified weighted Deming's method (8), a procedure appropriate for correlating results when both variables are subject to error. The weighting function used was $w_i = [(x_i + y_i)/2]^{-2}$. Since only single determinations were made and the methodology utilized for both x and y variables was identical, the value of λ was assumed to $= 1$, where λ is the ratio of error variances of the x and y measurements. Both the fitted line, $y = mx + b$, and the t-statistic for the slope (versus unity) were computed. The t statistic was compared to two-tail t values at $p < 0.05$ and $p < 0.01$ for significance. The coefficient of determination, r^2, for the fitted line was also obtained. The number of samples per laboratory varied depending on the number of months of program participation. For regression purposes, proficiency samples and cross-check samples assaying less than the reportable LOQ (PL = 0.2, WB = 2.0 ng/ml) or greater than the reportable HOQ (PL = 5, WB = 80 ng/ml) were omitted. Validation samples assaying outside of these limits were also omitted.

RESULTS

Validation Program

Between March 1991 and September 1992, 16 sites completed validation steps for whole blood

analysis and 11 of these also completed validation steps for plasma analysis. Eleven laboratories monitoring hepatic transplant patients initially utilized the plasma EIA; whole blood monitoring was performed simultaneously in a limited capacity for this population, starting in 1992 until the Abbott IM_x (9) was adopted by nearly all sites in November 1992. The six laboratories monitoring renal and bone marrow patients completed validation steps for whole blood analysis only.

Failures occurred at nearly all phases of the validation program for both plasma and whole blood analyses. Since most sites completed validation of plasma analysis first, failures were more common for this phase. Overall, 38% (6/16) sites initially failed part I, 4% (1/27) failed part II, 22% (6/27) failed part III, 30% (8.27) failed part IV, and 11% (3/27) failed part V. (NB: n = 27, 11 sites for plasma + 16 sites for whole blood.) Laboratories simply repeated the failed part of the validation phase until satisfactory results were obtained. Results compiled in this report reflect final outcomes achieved at each laboratory.

Intra- and interassay means and CVs for controls are compiled for whole blood (Table 1) and plasma (Table 2). FCL results are shown for comparison. Intraassay variability ranged from 2 to 29% for whole blood and from 5 to 27% for plasma. Interassay variability ranged from 5 to 27% for whole blood and from 7 to 30% for plasma. Variability within and between assays was greater at lower FK 506 concentrations than at medium or high FK 506 concentrations. Notably, the intraassay CV exceeded the interassay CVs of several sites; the CV from the reference laboratory, in one case, exceeded those of the other sites.

Figure 3 and Tables 3 and 4 display results for whole blood and plasma unknowns utilized for part V of the validation process. Resulting overall slopes were fairly close to 1:1.01 for whole blood and 1.13 for plasma. Intercepts were insignificant and well below the LOQ for the assays: whole blood = −0.23 (LOQ = 2.0 ng/ml) and plasma = −0.06 (LOQ = 0.2 ng/ml). Agreement between FCL and site values was good: r^2 = 0.83 for 836 whole blood samples and r^2 = 0.90 for 497 plasma samples.

Slopes from 56% (9/16) of laboratories assaying whole blood unknowns were significantly different from unity at the 95% confidence level (two-tail); 45% (5/11) of laboratories assaying plasma unknowns also had slopes significantly different from 1. Overall, whole blood unknowns from all sites yielded a slope (1.01) not significantly different from the FCL values; however, the slope (1.13) from plasma unknowns from all sites tested as significantly different from 1.

TABLE 1. *Summary of validation precision for FK 506 EIA assay: whole blood*

FK 506 concentration (ng/ml) Clinical site	Intraassay mean (CV)			Interassay mean (CV)		
	4.0 (Low)	15.0 (Med)	60.0 (High)	4.0 (Low)	15.0 (Med)	60.0 (High)
1[a]	4.0 (11.8)	14.4 (12.2)	62.1 (10.6)	4.0 (17.7)	14.4 (17.4)	63.9 (17.3)
2	3.8 (18.2)	14.1 (13.1)	73.9 (23.6)	4.7 (7.0)	15.3 (14.5)	65.3 (14.8)
3	4.4 (8.2)	14.6 (7.0)	62.9 (10.9)	4.1 (16.1)	15.3 (11.1)	70.9 (13.8)
4	4.0 (17.7)	14.2 (11.2)	60.6 (24.4)	4.9 (15.5)	15.3 (13.8)	52.7 (13.5)
5	4.0 (24.0)	13.7 (17.0)	58.7 (11.0)	4.2 (18.5)	15.0 (9.4)	63.1 (13.0)
6	3.9 (26.1)	17.5 (17.4)	61.1 (18.0)	4.6 (12.5)	14.8 (13.6)	70.3 (7.7)
7	4.0 (13.4)	17.2 (8.3)	63.7 (4.4)	4.1 (9.6)	15.3 (12.0)	57.3 (7.8)
8	4.9 (28.7)	16.7 (7.8)	64.6 (1.5)	4.1 (18.8)	16.3 (11.6)	63.2 (5.7)
9	3.7 (10.3)	12.0 (7.8)	55.4 (12.9)	4.6 (12.8)	14.3 (7.5)	49.5 (15.6)
10	3.8 (9.8)	13.7 (17.6)	60.7 (9.6)	3.7 (19.2)	14.5 (14.1)	61.2 (14.1)
11	3.8 (10.9)	14.0 (11.4)	45.7 (6.5)	4.1 (11.0)	15.6 (12.0)	50.0 (11.0)
12	3.3 (9.2)	17.1 (4.3)	43.7 (4.4)	4.6 (6.3)	17.5 (15.5)	51.6 (14.2)
13	3.6 (8.0)	17.9 (7.0)	65.1 (3.4)	4.6 (23.1)	17.3 (11.7)	69.1 (11.8)
14	4.4 (8.4)	16.5 (10.6)	61.3 (18.0)	4.0 (10.7)	14.5 (7.1)	63.2 (4.5)
15	4.3 (13.8)	11.7 (9.9)	58.7 (15.8)	4.2 (27.0)	13.6 (19.0)	57.4 (17.0)
16	4.0 (11.4)	15.6 (7.5)	61.2 (7.5)	4.2 (13.2)	15.4 (17.1)	56.8 (10.1)
Site mean (CV)	4.0 (14.4)	15.0 (10.6)	60.0 (11.4)	4.3 (14.9)	15.3 (13.0)	60.3 (12.1)
FCL mean[b] (CV)	3.9 (18.0)	14.0 (6.6)	73.6 (6.0)	4.2 (21.0)	16.8 (19.6)	66.1 (14.9)

[a] Mean of two technicians.
[b] 7 assay days.

TABLE 2. *Summary of validation precision for FK 506 EIA assay: plasma*

FK 506 concentration (ng/ml) Clinical site	Intraassay mean (CV) 0.3 (Low)	1.2 (Med)	3.0 (High)	Interassay mean (CV) 0.3 (Low)	1.2 (Med)	3.0 (High)
1	0.3 (12.4)	1.2 (5.6)	3.2 (9.5)	0.3 (13.7)	1.2 (10.4)	3.1 (7.9)
2	0.3 (21.6)	1.0 (16.1)	2.9 (6.8)	0.3 (29.6)	1.2 (11.8)	3.0 (8.0)
3	0.3 (20.7)	1.3 (12.0)	3.3 (5.8)	0.3 (27.5)	1.1 (13.6)	2.8 (15.2)
4	0.3 (27.4)	1.4 (25.6)	3.7 (15.2)	0.3 (27.5)	1.1 (15.2)	3.1 (13.9)
5	0.4 (19.4)	1.5 (15.1)	3.3 (7.5)	0.3 (9.7)	1.1 (22.3)	2.7 (13.3)
6	0.2 (17.2)	0.9 (11.9)	2.6 (8.1)	0.3 (9.5)	1.1 (12.3)	3.0 (6.6)
7	0.3 (20.5)	1.1 (15.8)	3.1 (15.0)	0.3 (11.1)	1.3 (21.6)	3.4 (21.5)
8	0.3 (15.8)	1.3 (9.3)	3.5 (9.6)	0.3 (18.1)	1.2 (11.5)	3.1 (21.2)
9	0.3 (16.0)	1.1 (5.4)	3.1 (10.0)	0.3 (24.1)	1.4 (9.6)	3.3 (17.0)
10	0.3 (8.5)	1.5 (13.2)	3.5 (11.7)	0.3 (19.4)	1.2 (8.6)	3.4 (11.2)
11	0.4 (22.0)	1.1 (11.0)	2.5 (8.0)	0.3 (24.0)	1.3 (22.0)	3.1 (18.0)
Site mean (CV)	0.3 (18.3)	1.2 (12.8)	3.2 (9.7)	0.3 (19.5)	1.2 (14.4)	3.1 (14.0)
FCL mean[a] (CV)	0.4 (9.7–11.1)	1.1 (5.7–7.6)	2.7 (7.7–11.0)	0.3 (16.3)	1.1 (12.8)	2.6 (9.0)

[a] n = 14 assays for interassay variability.

NATIONAL QUALITY ASSURANCE PROGRAM

Proficiency Samples

Tables 5 and 6 each provide a summary of regression analyses for proficiency sample assays of FK 506 for other site versus FCL whole blood and plasma results accumulated over 16 months (July 1991–October 1992). Figure 4 displays the agreement between the FCL and sites for all data collected.

Whole Blood

Agreement between FCL and other laboratory whole blood proficiency values (Table 5) was excellent, with r^2 ranging from 0.90 to 1.00. Slopes ranged from 0.90 to 1.12 and intercepts were insignificant or below the LOQ (2.0 ng/ml) for all sites. Only two sites had slopes that differed significantly from unity.

When all site values were compared to FCL target values via regression analysis, agreement was excellent (r^2 = 0.92), yielding the regression: other sites = 0.99 × FCL + 0.21, where n = 389. Overall, the slope was not significantly different from unity.

Plasma

The FCL and other-site plasma proficiency values (Table 6) agreed well as r^2 ranged from 0.81 to 0.96. Slopes ranged from 0.88 to 1.13 and all intercepts were insignificant and below the LOQ (0.2 ng/ml). Only one slope (site 10) tested significantly different from unity.

When all site values were compared to FCL target values using regression analysis, agreement was good (r^2 = 0.89), yielding: other sites = 1.00 × FCL − 0.02, where n = 430. The slope was statistically identical to unity.

Intraassay and Interassay Variation Assessments

Table 7 compares variation measures for each site for proficiency sample assays that were incorporated into the quality assurance program. Almost all sites performed within the expected range (CV ≤30%). Blank samples and samples yielding values greater than assay HOQ were in agreement as well.

Monthly Cross-Checks

Tables 8 and 9 display regression analysis results for other-site versus FCL whole blood and plasma cross-check specimens accumulated over 16 months (July 1991–October 1992). As displayed in Fig. 5, most patient samples measured within the lower half of the standard curve range: ≤2.5 ng/ml for plasma and ≤40 ng/ml for whole blood.

Whole Blood

Agreement was reasonable (r^2 = 0.62 − 0.97) between the FCL and 12 of the 15 sites comparing whole blood specimen results (Table 8). Slopes ranged from 0.79 to 1.18 and 10 of the 12 intercepts

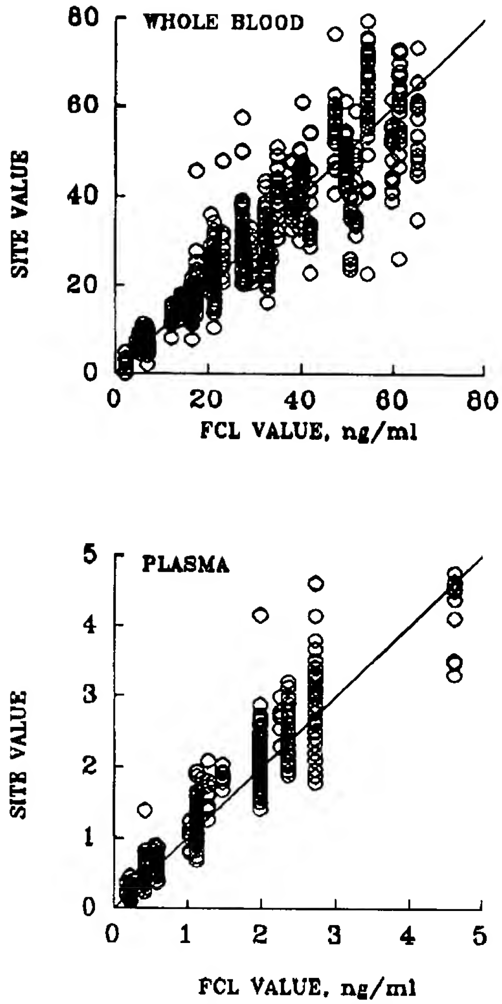


FIG. 3. Comparison of FK 506 concentrations measured in validation samples for whole blood and plasma for all participating laboratories versus the FCL. Line indicates slope of unity.

are insignificant and below the LOQ. Three sites showed poor correlations (r^2 = 0.15–0.40) and deviant slopes (1.31, 1.33, and 5.29), as well as intercepts greater than the LOQ (−2.39 and −20.70). Slopes from two of the 16 sites tested significantly different from unity.

Overall regression analysis yielded results that indicate excellent comparability: other sites = 1.03 × FCL + 0.02, where n = 478. Agreement was reasonable: r^2 = 0.75. This slope was not significantly different from unity.

Plasma

Degree of agreement between the FCL and 11 sites was less variable for reanalysis of plasma specimens, ranging from r^2 = 0.66 to 0.98 for 10 of the 11 sites (Table 9). Individual slopes ranged from 0.90 to 1.34. None of the 11 intercepts were significantly above the LOQ. The correlation for one site was poor (r^2 = 0.33). Slopes from four sites were significantly different from unity.

As shown in Fig. 3, overall regression analysis yielded results suggesting slightly lower FK 506 concentrations of jointly analyzed patient specimens on second analysis: other sites = 1.14 × FCL − 0.02, where n = 680. Agreement was reasonable: r^2 = 0.74. The slope generated was significantly different from unity.

DISCUSSION

The EIA method was the simplest, most sensitive methodology available for clinical use in monitoring patient therapy with FK 506 when initial clinical trials began. Subsequently, high-performance liquid chromatography (HPLC) with fluorescent derivatization of FK 506 has been used in limited pharmacokinetic studies in Japan (10). In the U.S.A., Abbott Laboratories developed an IM_x method for whole blood, employing the same antibody as that employed in the EIA (9). As of November 1, 1992, most clinical sites utilize this method for monitoring FK 506 concentrations. The disadvantages of the original EIA method include potential measurement of metabolites due to cross reactivity with the monoclonal antibody (11), length of time required to produce assay results (24–36 h), and the large variation experienced at the lower concentration range (CV up to 30% within one laboratory) (4). The latter is a common problem with EIA assays. These factors, along with concerns about adsorption of FK 506 to glass and plastics, argued for establishing a quality assurance program with one source of standards, common operating procedures, and interlaboratory standards for comparisons.

Common standards, controls, and primary reagents were supplied by Fujisawa and the FCL. Standards and controls were prepared, tested, and distributed by the FCL. Primary reagents, such as polyclonal antibody (IncStar), monoclonal antibody (Fujisawa Ltd), and FK 506-POD (Fujisawa Ltd) were in common usage. The standard operating procedure was identical for all laboratories. These commonalities eliminated potential additional sources of variation resulting from differences in methodology (12), calibrators (13), and measurements due to inconsistent nonspecific and cross-

TABLE 3. *Comparison of whole blood EIA for FK 506 validation result*

Clinical site	Slope (m)	y-intercept	t for $m \neq 1$	r^2	n	Proficiency score[a]
1	0.99	0.22	−0.35	0.88	53	93
2	1.02	1.41	0.43	0.87	56	87
3	1.22	0.56	5.96[c]	0.93	50	80
4	1.10	−0.09	1.34	0.85	57	72
5	1.08	−0.24	1.05	0.88	50	76
6	0.71	1.33	−7.19[c]	0.83	46	80
7	1.08	−0.57	2.10[b]	0.88	56	90
8	1.11	−0.70	1.78	0.89	57	83
9	1.10	0.12	2.28[b]	0.97	53	89
10	1.14	−1.13	3.77[c]	0.90	56	93
11	1.08	0.32	2.14[b]	0.97	50	82
12	0.85	0.39	−4.26[c]	0.89	46	93
13	0.96	−0.38	−1.08	0.85	45	86
14	0.80	0.28	−5.29[c]	0.86	50	86
15	1.36	−1.94	2.14[b]	0.97	57	80
16	1.04	0.34	1.09	0.94	53	100
All	1.01	−0.23	0.99	0.83	836	

[a] Percent of samples differing by ≤30% from nominal values.
[b] Significant at $p \leq 0.05$.
[c] Significant at $p \leq 0.01$.

reactive binding. Hence, the only differences existing between laboratories were those of technical performance and some minor items of equipment.

Validation Program

Technical training, advice, and support was provided by one "referee" laboratory, the FCL. This allowed for efficient central organization and consistency of technical support. Since the variability of this EIA was greater than desirable and the method tedious, large variation in results was expected. As the validation program was partly a learning experience for outside sites, larger variation in results was expected for unknowns (part V) than for proficiency assays performed in the quality assurance program. Last, the largest variation was expected for comparison of patient sample measurements since most patient concentrations fell closest to the lower control range, where variation was the highest.

The validation program sought to establish independent efficient operation of the FK 506 EIA at all laboratories without compromising quality. Results provided in this report show that each laboratory accomplished the task with different levels of proficiency; however, all laboratories attained suitable precision (and accuracy, which was not reported) below the 30% allowable limit. As noted in the results, the intraassay CV reported sometimes ex-

TABLE 4. *Comparison of plasma EIA for FK 506 validation results*

Clinical site	Slope (m)	y-intercept	t for $m \neq 1$	r^2	n	Proficiency score
1	1.21	−0.10	3.67[a]	0.88	45	83
2	1.04	0.00	0.66	0.89	50	95
3	1.05	−0.04	0.79	0.94	46	88
4	1.07	−0.09	1.22	0.92	44	90
5	1.11	−0.08	1.37	0.74	46	80
6	0.96	−0.03	−1.90	0.98	46	95
7	1.18	−0.06	3.08[a]	0.93	46	82
8	1.31	−0.10	12.0[b]	0.98	46	82
9	1.21	−0.12	5.52[a]	0.96	37	94
10	0.91	0.03	4.13[a]	0.94	46	91
11	0.99	−0.05	−0.14	0.95	45	75
All	1.13	−0.06	7.56[a]	0.90	497	

[a] Significant at $p \leq 0.01$.

TABLE 5. *Comparison of whole blood EIA for FK 506 proficiency results: July, 1991–October, 1992*

Clinical site	No. mo	Slope (m)	y-intercept	t for $m \neq 1$	r^2	n
1	9	0.96	0.74	−1.03	0.95	18
2	9	1.07	−0.38	0.98	0.90	17
3	1	0.99	1.88	−0.06	1.00	3
4	8	0.92	−0.03	−1.61	0.91	24
5	7	1.06	−0.42	1.39	0.96	21
6	15	0.95	0.51	−1.62	0.92	43
7	7	1.12	−0.26	2.64[a]	0.94	21
8	8	0.98	1.10	−0.45	0.95	23
9	9	0.95	−0.15	−1.27	0.94	26
10	7	0.91	1.89	−1.71	0.96	21
11	10	—	—	—	—	0
12	16	1.01	0.29	0.16	0.91	45
13	16	0.98	0.87	−0.58	0.92	45
14	14	0.95	0.27	−1.09	0.91	39
15	8	1.00	0.28	−0.43	0.94	23
16	7	0.90	−0.41	−2.91[b]	0.96	21
All	—	0.99	0.21	−1.21	0.92	389

[a] Significant at $p \leq 0.05$.
[b] Significant at $p \leq 0.01$.

ceeded the interassay CV. This is partly explained by the fact that the validation program was a learning process. Intraassay values achieved were the results of a second phase in performing the assay whereas interassay values came after early experience was gained (see Fig. 1). Values reported for the reference laboratory were also achieved early in establishing the validity of the assay.

Regression analysis of the validation unknowns indicated that a high percentage of the regression slopes from various sites differed significantly from 1.0. Such deviations, while indicative of a systematic difference between laboratories, are often not meaningful in terms of overall assay comparability. Occurrence of a lower r^2, indicating greater variability, diminishes the t value (e.g., Table 9, sites 4 and 10). The original criteria for success with the assay called for errors $\leq \pm 30\%$ of FCL values for 70% of samples. All three measures must be considered jointly for proper assessment of comparability of results from different laboratories.

The minimum time required to complete the validation procedure is 3 weeks. Most clinical laboratories were operational within 1–2 months following acquisition of equipment and commitment to the program.

Quality Assurance Program

The quality assurance program was voluntarily established by Fujisawa and the FCL in a collaborative effort, rather than having been mandated by

TABLE 6. *Comparison of plasma EIA for FK 506 proficiency results: July, 1991–October, 1992*

Clinical site	No. mo	Slope (m)	y-intercept	t for $m \neq 1$	r^2	n
1	14	1.04	−0.09	0.70	0.90	39
2	12	0.96	0.02	0.97	0.91	33
3	14	1.05	−0.09	0.53	0.86	38
4	15	0.96	0.03	−0.86	0.91	41
5	15	0.98	0.01	−0.33	0.96	42
6	16	0.88	0.09	−1.34	0.93	45
7	15	1.03	−0.01	0.52	0.90	42
8	15	0.94	0.14	−0.63	0.81	42
9	15	0.98	−0.10	−0.20	0.95	42
10	11	1.13	−0.01	2.23[a]	0.93	30
11	13	0.99	0.03	−0.22	0.93	36
All	—	1.00	0.02	0.14	0.89	430

[a] Significant at $p \leq 0.05$.

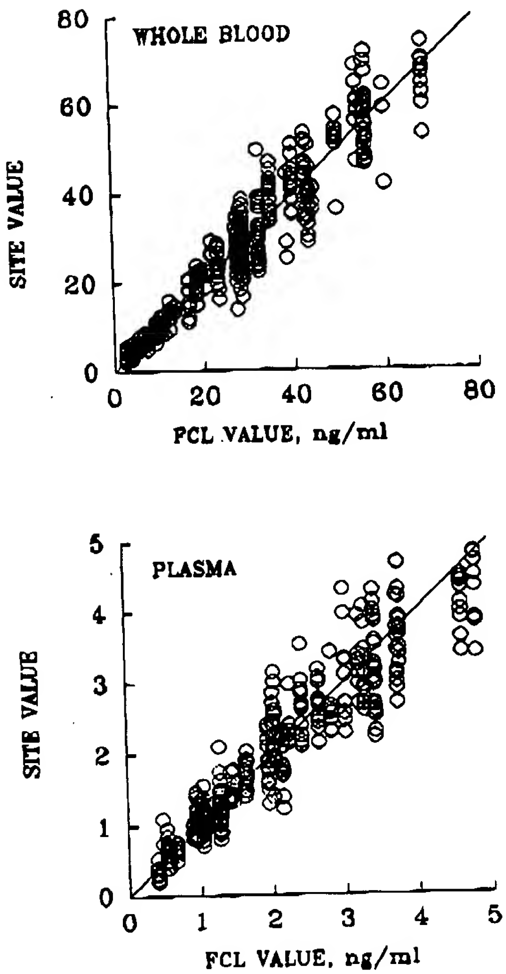


FIG. 4. Comparison of FK 506 concentrations measured in proficiency samples in whole blood and plasma for all participating laboratories versus the FCL. Line indicates slope of unity.

the Food and Drug Administration (FDA). The goals were to reduce interlaboratory variation and eliminate the need to later adjust or standardize compiled multicenter FK 506 data for New Drug Application (NDA) submission (14). In order to achieve and validate these goals, participation by each clinical site in this program was mandatory. Overall, this program yielded excellent agreement in FK 506 concentrations between laboratories and the FCL. However, several laboratories performed less satisfactorily than others.

Proficiency samples yielded the most impressive correlations. Slopes were fairly close to 1.0 and correlation coefficients near 0.90 in interlaboratory comparisons (Fig. 3). Individually, each laboratory performed proficiency testing well within the limitations of the assay. Intra- and interassay measures assessed in late 1991 produced acceptable results in 93% (14/15) of laboratories. Since proficiency samples were tested numerous times at the FCL, and stored and shipped under identical conditions, this quality assurance measure was the most objective of all comparisons made in the program.

Cross-check samples emphasized the lower FK506 concentrations, where most patient results were likely to occur, but where variability of the assay was highest (4). Thus, correlations were poorest for this quality assurance measure. Cross-check results were satisfactory at most sites, but indicated that FK 506 had possibly degraded slightly in plasma, since slopes were >1.0 in most, but not all, laboratories. The correlation was adequate ($r^2 \geq 0.7$) for 92% (11/12) of laboratories analyzing plasma and for 73% (11/15) of laboratories assaying whole blood. Sites exhibiting poorer correlation for cross-check samples always exhibited better results for proficiency samples. Degradation of cross-check samples might have occurred naturally or been caused by inconsistencies in specimen processing, i.e., amount of time kept at room temperature, number of freeze-thaw cycles, or storage. A stricter sample control process for this part of the program was clearly indicated.

A further comparison of interlaboratory data, i.e., examining regression slopes for statistically significant differences, was reviewed. Generally,

TABLE 7. *Intra- and interassay variability tested in FK 506 quality assurance program as proficiency samples*

Clinical site	Intraassay CV	Interassay CV
Whole blood		
1	35.39	7.32
2	17.10	13.80
3	8.56	11.19
4	1.05	23.98
FCL	6.46	18.30
Plasma		
1	10.79	28.55
2	10.70	26.55
3	0.63	15.31
4	8.71[a]	9.59
5	5.41	17.00
6	2.52	11.68
7	26.94	36.55
8	4.95	3.79
9	12.26[a]	16.84
10	14.98	9.08
11	3.22	2.65
FCL	8.36	9.76

[a] Mean of two technicians.

TABLE 8. *Summary of results of cross-check analysis for whole blood FK 506 EIA: July, 1991–October, 1992*

Clinical site	No. mo	Slope (m)	y-intercept	t for $m \neq 1$	r^2	n
1	5	1.15	−2.00	1.36	0.90	24
2	3	0.85	−0.00	−3.28[a]	0.97	16
3	0	—	—	—	—	0
4	3	1.33	−2.39	1.22	0.39	17
5	4	1.14	−0.72	1.54	0.62	19
6	10	1.09	0.72	1.04	0.89	49
7	1	5.29	−20.70	0.13	0.15	5
8	7	0.79	2.66	−3.65	0.87	33
9	10	0.91	−0.06	−1.84	0.82	49
10	7	1.31	−0.40	1.57	0.40	35
11	0	—	—	—	—	0
12	14	0.97	−0.33	−0.40	0.84	73
13	16	1.08	−0.21	1.31	0.80	79
14	6	0.84	1.75	−2.05	0.75	30
15	7	1.18	−0.56	2.76	0.83	33
16	4	0.98	0.53	−0.20	0.84	15
All	—	1.03	0.02	1.36	0.75	478

[a] Significant at $p \leq 0.01$.

most sites and overall measures tested well, with the exception of the plasma cross-check portion. However, once again, we believe that the t-test overweighs the significance of the differences, given the high variation inherent in the methodology.

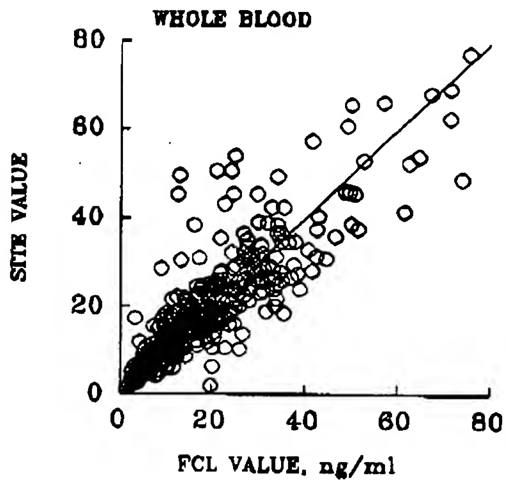
Differences between laboratories and types of comparisons may also be due to a number of variables. The number of analyses performed at the FCL differed; proficiency targets were established from a mean of six or more values whereas cross-check assay of specimens were performed one time only. Proficiency samples were prepared by adding pure FK 506 to normal pooled plasma for processing and distribution by one laboratory (the FCL). Cross-check specimens, representing various patients on FK 506 therapy at 16 different clinical sites, were processed in normal fashion by their laboratories. Minor inconsistencies in specimen handling between laboratories, coupled with inherent variability in patient biochemistry and pharmacokinetics, metabolite formation, and concomitant therapies could cause cross-check specimens to be subject to more error.

During the first year of this program, FCL values were utilized as reference values. This approach seemed logical, as the FCL had established the methodology and used it for some time, and had provided training and support to other laboratories. However, once other sites were operational for an

TABLE 9. *Summary of results of cross-check analysis for plasma FK 506 EIA: July, 1991–October, 1992*

Clinical site	No. mo	Slope (m)	y-intercept	t for $m \neq 1$	r^2	n
1	14	1.15	0.02	1.30	0.66	68
2	9	1.01	−0.01	0.11	0.79	39
3	14	1.17	0.02	2.17[a]	0.84	61
4	14	0.90	0.07	−0.63	0.33	80
5	15	1.12	−0.06	1.51	0.98	73
6	13	0.96	−0.04	−0.56	0.77	56
7	14	1.34	−0.05	2.94[b]	0.87	63
8	14	1.07	−0.03	0.75	0.80	63
9	15	1.20	−0.05	2.96[b]	0.77	75
10	11	1.28	−0.06	1.65	0.69	50
11	13	1.18	0.04	2.05[a]	0.92	54
All	—	1.14	−0.02	4.49[b]	0.74	680

[a] Significant at $p \leq 0.05$.
[b] Significant at $p \leq 0.01$.



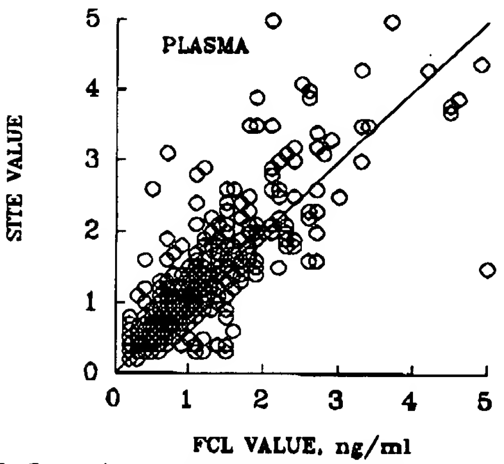


FIG. 5. Comparison of FK 506 concentrations measured in cross-check samples of whole blood and plasma for all participating laboratories versus the FCL. Line indicates slope of unity.

extended period, using the resulting peer group means may be of advantage. While Teitz et al. (15) argued against use of peer group means for proficiency testing, use of this tool for this particular program may well be the best alternative, as there is no reference method available for FK 506. Also, preparation, testing, and distribution of large batches of reference material for a small number of laboratories is laborious.

Acknowledgment: Laboratories/transplant centers at the following sites participated in the FK 506 monitoring program: Baylor University Medical Center (Dallas, TX, U.S.A.), Mayo Clinic (Rochester, MN, U.S.A.), University of Nebraska Medical Center (Omaha, NE, U.S.A.), University of California at San Francisco (San Francisco, CA, U.S.A.), University of California at Los Angeles (Los Angeles, CA, U.S.A.), California Pacific Medical Center (San Francisco, CA, U.S.A.), Barnes Hospital Washington University (St. Louis, MO, U.S.A.), Mount Sinai Medical Center (New York, NY, U.S.A.), New England Deaconess (Boston, MA, U.S.A.), University of Chicago (Chicago, IL, U.S.A.), Johns Hopkins Hospital (Baltimore, MD, U.S.A.), University of Alabama (Birmingham, AL, U.S.A.), Fred Hutchinson Cancer Center (Seattle, WA, U.S.A.), Emory University (Atlanta, GA, U.S.A.), University of Minnesota (Minneapolis, MN, U.S.A.), and St. Vincent's Hospital (Los Angeles, CA, U.S.A.). Staff of the FCL providing technical and clerical assistance includes Fung Sing Chow, Theresa Crissy, Hon Yi Yuen, Suzette Mis, Kimberly Moore, Patricia Rackl, Karin Riedl, Stephen B. Rogenthein, Deneice Stetz, Freddie Wang, and Sandra M. Wheaton.

We appreciate the support of Dr. Masakazu Kobayashi and assistance of Tracy Orlando of the Fujisawa Pharmaceutical Company. Dr. Kristian Linnet kindly supplied his version of the weighted Deming's procedure (8). Programming assistance was provided by Mr. Yiming Gong

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FK 506 Assay Past and Present—Characteristics of FK 506 ELISA

M. Kobayashi, K. Tamura, N. Katayama, K. Nakamura, K. Nagase, K. Hane, T. Tutumi, M. Niwa, H. Tanaka, K. Iwasaki, and M. Kohsaka

FK 506 is a 27-ring macrolide antibiotic with strong immunosuppressive activity. Its chemical and crystallographic structures have been reported by H Tanaka et al[1] and T Taga et al,[2] respectively. With its strong immunosuppressive activity, FK 506 is now in multicenter clinical trials for kidney and liver transplantations in Europe, the United States, and Japan. Like cyclosporine (CyA), drug level monitoring is an important issue. By comparing its activity with CyA and CyA's blood concentration (whole blood at 300 ng/mL and plasma at 50 ng/mL), development of an assay method with minimal detection levels of approximately 0.05 to 0.5 ng/mL for plasma and 0.3 to 3 ng/mL for whole blood were expected. From its structure, it was evident that FK 506 does not have a strong chromophore in the molecule and it was considered inappropriate to use the high performance liquid chromatography-ultraviolet detector (HPLC-UV) method. While the detection limit was 50 ng/mL for pure FK 506 with HPLC-UV, the detection limit was increased to 100 ng/mL with the spiked sample in dog plasma.

With the availability of a monoclonal antibody (MAb) to FK 506, we established a double antibody enzyme-linked immunosorbent assay (ELISA) for FK 506[3] that has been used for therapeutic monitoring in organ transplantation at the University of Pittsburgh since 1989 with the Sep-Pak extraction method for plasma.[4] Like CyA, FK 506 is present in bound form in red blood cells (RBCs) in whole blood[5]; an assay method for whole blood was therefore desirable. Since the introduction of the ELISA method, the main concern has been the cross-reactivity of possible metabolites in the ELISA.[6,7] Improvement of sensitivity by the HPLC method has been tried for a specific assay using chemiluminescence detection[8] or in conjunction with the Fab mass detector.[9] Metabolites that work with the improvement of the detection method should afford more appropriate analysis for therapeutic monitoring. However, in the light of clinical benefit currently ongoing with the drug level, we will focus our discussion on the characteristics of the ELISA in this paper. We report a unified extraction method for whole blood and for plasma, and correlation for the Sep-Pak method. Furthermore, the characteristics of the ELISA were thoroughly analyzed. From the preliminary analysis of mixed lymphocyte reaction (MLR) suppressive and immunocross-reactivities of FK 506 analogues and metabolites, the validity of the ELISA for patients' immunosuppressive state was partly suggested and a possible epitope on FK 506 to MAb was proposed.

METHODS AND RESULTS

Antimouse IgG (Cat #83605) was purchased from Atlantic Antibodies (Stillwater, MN, USA).

Comparison of the Extraction Method for FK 506 Concentration With Spiked Human Plasma Obtained From Kidney Transplant Patients

The Sep-Pak extraction method for plasma specimen has been reported by the Pittsburgh group.[4]

CH_2Cl_2 Liquid–Liquid Extraction for the Plasma Specimen. One hundred microliters of the plasma sample was added to a mixture of 0.1 mol/L phosphate buffer (pH 7.0) 1.0 mL, CH_2Cl_2 5.5 mL, and 10 μL of MeOH. The mixture was shaken vigorously (200 strokes/min) and centrifuged at 3,000 rpm for 10 minutes. The aqueous layer was aspirated, and 4.5 mL of the CH_2Cl_2 layer was transferred to a glass tube to which N_2 gas stream was introduced for a concentration below 37°C. The dried residue was reconstituted in 180 μL of 300-fold diluted peroxidase-labeled FK 506 (FK-POD) solution in BT-phosphate-buffered saline (PBS) (1% bovine serum albumin [BSA], 0.05% Tween 20-PBS) and 150 μL was used for the FK 506 ELISA.

CH_2Cl_2 Liquid–Liquid Extraction for Whole Blood. Ten microliters of the whole blood sample was used instead of plasma (100 μL) and extracted into 5.5 mL of CH_2Cl_2 by the same method as described above.

With the spiked plasma sample, both extraction methods gave anticipated values as shown in Table 1. However, with patient specimens the Sep-Pak method tends to give approximately a twofold higher value than the CH_2Cl_2 extraction method. The correlation coefficient was 0.91 for 30 Japanese kidney transplant samples (plasma trough data) and 10 European liver transplant samples (plasma data) in clinical trials (Fig 1).

FK 506 Recovery Dependence on the Extraction Period

^{3}H-dihydro-FK 506 (sp. act. = 50.3 Ci/mmol; 52,608 dpm in MeOH 10 μL) was extracted according to the plasma CH_2Cl_2

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Table 1. Precision and Accuracy of the Sep-Pak Method for Plasma Spiked Samples and of the CH_2Cl_2 Extraction Method for Plasma and Whole Blood Spiked Samples

Extraction	Sample	Sample Volume (μL)	Concentration (ng/mL) Added	Found	Accuracy (%)	Precision (CV%)
Sep-Pak	Plasma	100	0.05	0.044	88.0	17.65
	(dog)	100	0.25	0.260	104.0	7.53
		100	3.0	2.960	98.7	6.41
CH_2Cl_2	Plasma	100	0.30	0.32	107	6.0
(n = 5)	(human)	100	1.0	1.0	100	7.4
		100	3.0	2.8	93	11.3
	Whole	10	3.0	3.0	100	10.6
	blood	10	10	11	110	9.6
	(human)	10	30	33	110	4.7
	Whole	100	0.30	0.31	103	20.1
	blood	100	1.0	1.1	110	14.0
	(human)	100	3.0	3.3	110	12.9

Abbreviation: CV, coefficient of variance.

extraction method as described above. Extraction was stopped at the designated time, and the radioactivity in the CH_2Cl_2 layer was counted, from which the recovery yield was calculated.

The results of time dependency on extraction yield into CH_2Cl_2 are shown in Fig 2. After 30 minutes of extraction, the yield plateaus at 85%.

Stability of FK 506 in MeOH

Ten μg/mL of FK 506 in MeOH was prepared and kept at −20°C. The sample was analyzed by normal phase HPLC using TSKgel ODS-80T M (4 × 150 mm) under an isocratic condition of

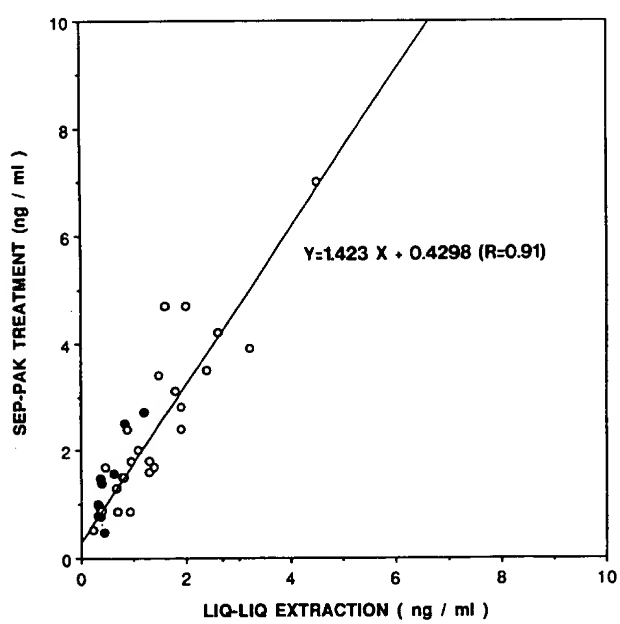


Fig 1. Correlation between Sep-Pak extraction and CH_2Cl_2 extraction for Plasma FK 506 level. Patients' plasma samples (N = 40) were extracted both by Sep-Pak and CH_2Cl_2 methods. The extraction was duplicate and ELISA was single for each extraction.

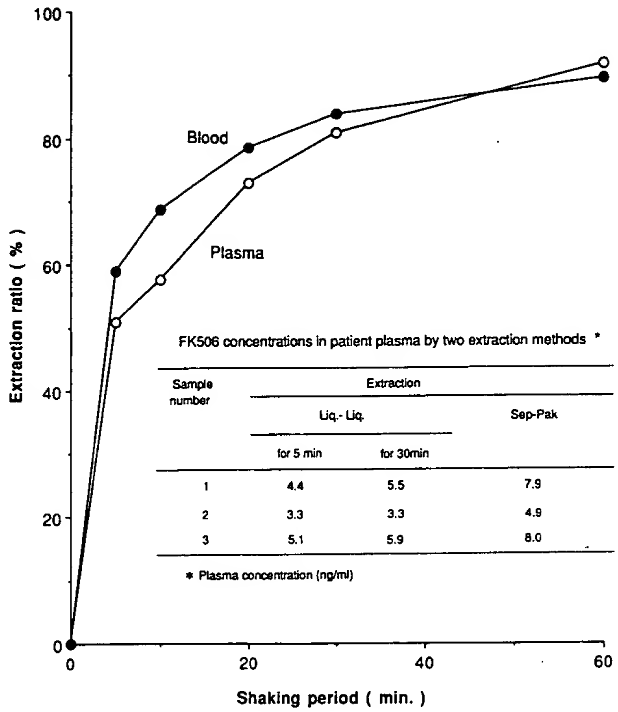


Fig 2. Time dependence of ^{3}H-dihydro-FK 506 into CH_2Cl_2 on the recovery yield.

H_2O:THF:MeOH (5:3:2) at a flow rate of 1 mL/min at a constant temperature of 50°C. Detection was by UV 220 nm. On the day of quantitation, a series of standard solutions were prepared freshly from FK 506 powder.

The results are summarized in Table 2. Although a rapid but small decrease in FK 506 concentration was noted in the first week, almost no loss of FK 506 was confirmed in the following days up until 8 months at −20°C.

Stability of FK 506 in Plasma and Whole Blood

A 100-fold concentrate of FK 506 in MeOH was prepared and mixed at 1:100 with human plasma or whole blood to prepare the FK 506 solution with the designated concentration. The prepared plasma and whole blood spiked specimens were kept at −20°C until analyzed by ELISA with the CH_2Cl_2 extraction method. In this stability test, −20°C stocked solution of FK 506 standard at 10 μg/mL in MeOH was used for quantitation.

The results are also shown in Table 3. Spiked FK 506 was stable at −20°C for 6 months in plasma and in whole blood.

Table 2. Stability of FK 506 in MeOH Assayed by HPLC

Concentration Quantitated (μg/mL)	Residual % Stored at −20°C for 0	1 wk	2 wk	4 wk	2 mo	4 mo	8 mo
8.96	100	95.5	95.3	95.2	94.4	95.6	95.9

Table 3. Stability of FK 506 in Plasma and in Whole Blood Assayed by ELISA

Concentration Added (ng/mL)	Concentration Detected (ng/mL) Stored at −20°C for 1 wk	6 wk	9 wk
Plasma			
1.0	0.90 ± 0.04	1.4 ± 0.0	1.0 ± 0.0
10.0	9.7 ± 0.9	11 ± 1	10 ± 0
Whole blood			
10	8.8 ± 0.1	11 ± 1	8.6 ± 0.3
100	90 ± 10	98 ± 1	86 ± 17

Preparation and Influence of RBC Lysate on the FK 506 ELISA

Human RBCs were collected from 25 mL of heparinized whole blood by gradient centrifugation over Mono-Poly resolving medium (M-RPM; Flow Labs). After several washings with PBS, RBCs were sonicated in distilled H_2O (total volume of 25 mL) for 5 minutes. Cell homogenate was cleared of cell debris by centrifugation at 20,000*g* for 30 minutes to afford RBC lysate. Protein concentration by the Lowry method was 137.6 mg/mL.

As is shown in Fig 3A, the shift by lysate was concentration dependent. When the protein concentration in the assay well was 2.75 mg/mL, no optical density (OD) response was observed throughout the FK 506 concentration tested (0.02 to 10 ng/mL). It was noteworthy in the titration curve that a higher OD was observed at higher FK 506 concentrations in the presence of the lysate, while at lower FK 506 concentrations maximum OD was suppressed, indicating the decreased binding of FK-POD to MAb on the solid surface.

Influence of α1-Acid Glycoprotein and Human FKBP on FK 506 ELISA

FKBP (334 μg) was purified from human tonsils (66.4 g) homogenate by a sequence of heat treatment-DEAE chromatography-ultrafiltration over XM-50 and YM-5-reversed phase HPLC, and its structure was confirmed by amino acid sequence. Binding of appropriately diluted tested protein with serially diluted FK 506 in an assay buffer was equilibrated for 30 minutes and the interference by the interaction to the ELISA was examined from the OD difference.

α1-Acid glycoprotein, which is considered to be a main plasma circulating protein with FK 506 binding property, did not show any influence on the FK 506 ELISA at as high as 1 mg/mL. Thus, this binding is considered to be nonspecific and simply by hydrophobic interaction, cannot compete with anti-FK 506 Mab to free FK 506 (Fig 3A).

On the other hand, FKBP as low as 1 μg/mL in the assay well completely inhibited the binding of FK-POD to MAb on the solid phase (Fig 3B). It was also noted that at a high FK 506 level, competitive binding of free FK 506 was suppressed, thus giving higher OD as was observed with the RBC lysate experiment (Fig 3B).

Influence of Other Comedicated Drugs Along with FK 506 to the FK 506 ELISA

Drugs tested were prednisolone (Wako Chemicals, Japan), fluconazole (Pfizer Pharmaceutical), azathioprine (Sumitomo Pharmaceutical, Japan), amphotericin B (Bristol Meyers-Squibb Co), gentamycin (Schering-Plough), and nifedipine (inhouse synthesized) at a final concentration of 100 nmol/L in the assay well, except for CyA, which was mixed with FK 506 at 200 (167 nmol/L) and 500 ng/mL (416 nmol/L).

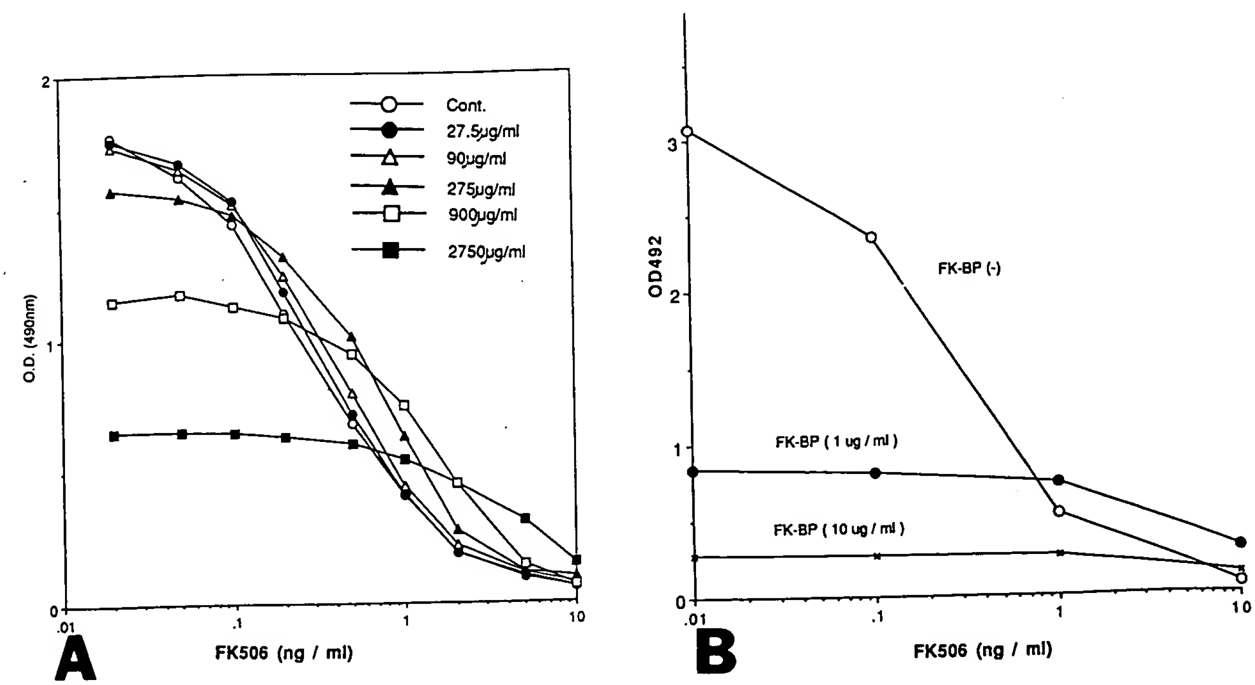


Fig 3. **(A)** The influence of human RBC lysate on FK 506 ELISA. **(B)** The influence of FKBP on FK 506 assay.

Table 4. The Influence of CyA at 200 and 500 ng/mL

FK 506 Sample (ng/mL)	Added CyA (ng/mL [nmol/L])	FK 506 Measured (ng/mL)	Recovery (%)
0.0739	200 (167)	0.0758	102.6
	500 (416)	0.088	119.2
0.384	200 (167)	0.411	107.0
	500 (416)	0.414	107.8
1.30	200 (167)	1.375	105.8
	500 (416)	1.370	105.4

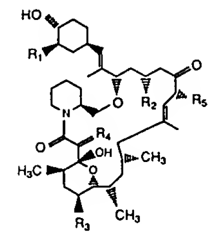
NOTE: CyA was mixed with FK 506 solution in assay buffer and added to the assay well.

Noninterference by CyA was confirmed up to 500 ng/mL (Table 4), and other drugs also showed no influence on the ELISA.

Immunocross-reactivity and MLR Suppressive Activities of FK 506 Derivatives and Two Metabolites

Binding by competition of serially diluted FK 506 metabolites and FK 506 derivatives to MAb was tested in the FK 506 ELISA system. Relative immunocross-reactivity was calculated from their IC_{50} (ng/mL).

Structures and correlation between MLR suppressive activity and immunocross-reactivity of FK 506 analogues and metabolites examined are shown in Fig 4. Relatively reasonable correlation was confirmed. One of the metabolites, FR901154 demethylated at C-31,[9] did show 76% immunocross-reactivity with 37.7% MLR suppressive activity. The second metabolite (M1) showed immunocross-reactivity and no MLR suppressive activity.[10]



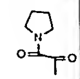
Compound	R_1	R_2	R_3	R_4	R_5	MLR	Immunocross-Reactivity
FK506	OCH_3	OH	OCH_3	=O		100%	100%
FR901156	—	—	—	—		62.2	50.0
FR900520	—	—	—	—		41.8	34.8
FR900523	—	—	—	—	CH_3	14.4	14.8
FR901154	OH	—	—	—		37.7	76.2
FR901155	=O	—	—	—		32.4	53.3
FR126724	—	H_2	—	—		28.0	2.7
FR126865	—	H_2	—	OH		N.T.	0
M1	—	—	OH	=O		0	0
FR900525 	—	—	—	—		16.8	3.1
rapamycin						—	0

Fig 4. Structures and correlation between MLR suppressive activity and immunocross-reactivity of FK 506 analogues and metabolites.

DISCUSSION

Following the establishment of the Sep-Pak method as an extraction method for plasma samples to remove ELISA-interfering substances, we established the CH_2Cl_2 method to replace Sep-Pak for both plasma and whole blood samples. It was anticipated that the CH_2Cl_2 extraction method might be more preferable in the sense that FK 506 and its metabolites can be extracted by the same method. Well precision for both spiked plasma and whole blood samples was established with CH_2Cl_2 extraction (Table 1). However, when patient plasma trough levels measured by Sep-Pak and CH_2Cl_2 extraction methods were compared, consistently higher values were observed with the Sep-Pak method (Fig 1). The correlation coefficient was 0.91 with 40 samples from Japan kidney transplant (30 samples) and European liver transplant samples (10). This indicates that as long as the same extraction method is used, similar information can be drawn although the absolute value is different. Currently, an automated assay system is under development by Abbott Labs.[11]

Extraction efficiency into CH_2Cl_2 from plasma was examined using ^{3}H-dihydro-FK 506. As is shown in Fig 2, the efficiency was time dependent, and after 30 minutes extraction, the recovery yield plateaued at 85% from both whole blood and plasma samples. When patient samples were measured with 5-minute and 30-minute extractions in which standard calibrators were treated the same way, respectively, the same concentration was obtained, suggesting that FK 506 and its metabolites in plasma extractable into CH_2Cl_2 were extracted in the same yield as that of FK 506 in the calibrators (table insert in Fig 2).

RBC lysate showed clear inhibition at 275 μg/mL of protein concentration in the well. Titration study showed a higher FK-POD binding at high free FK 506 and low FK-POD binding at low free FK 506, suggesting the presence of specific inhibitors in the lysate to free FK 506 antibody interaction. This was supported by the presence of FKBP-like protein in the RBC lysate.[10]

α-1-Acid glycoprotein, which was found as a main circulating binding protein for FK 506, did not show any influence on the FK 506 ELISA (Fig 3A) up to 1 mg/mL. Thus, the binding of α-1-acid glycoprotein is a nonspecific hydrophobic interaction, and FK 506 is easily captured by MAb from its binding to α-1-acid glycoprotein. Human FKBP was purified from human tonsillar cells and its effect was tested on the ELISA. FKBP, when added to the assay, strongly inhibited the binding of FK-POD to MAb on the solid phase at 1 μg/mL (Fig 3B). The pattern of inhibition is very similar to the one by the RBC lysate; high FK-POD binding at high free FK 506 and low FK-POD binding at a low free FK 506 concentration.

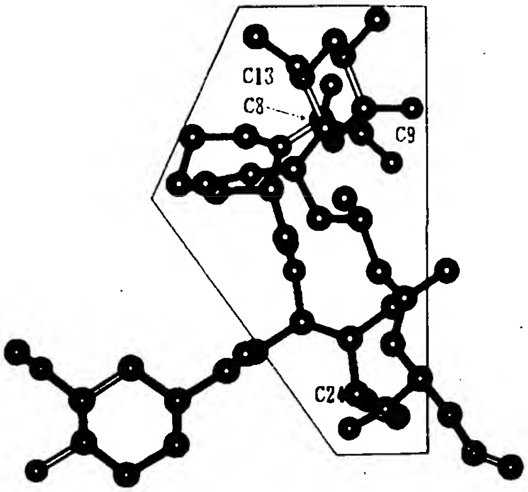


Fig 5. Postulated epitope of FK 506 to MAb.

When an interference on the FK 506 ELISA by other possible comedicated drugs was examined, CyA at 200 and 500 ng/mL did not show interference, and quantitative recovery was confirmed (Table 4). Prednisolone, fluconazole, azathioprine, amphotericin B, gentamycin, and nifedipine at 100 nmol/L did not interfere with the FK 506 ELISA at all (data not shown).

From the study of structures and immunocross-reactivities (Fig 4), we may postulate the epitope recognized by MAb to be a space formed by the C24-OH, C8-C9 α-dicarbonyl group, and C13-OMe group as shown in Fig 5. Examination of the FK 506 3D-structure[1] indicates that C24-OH and C13-OMe are directed in the opposite way and separated by 11.2 A. However, if FK 506 is rendered to conformational change as is the case of binding to FKBP,[12] all epitope-related residues are to be placed in a close vicinity. Good correlation between MLR immunosuppressive activity and immunocross-reactivity to MAb, especially C13-demethylated FK 506, M-1, with both no immunocross-reactivity to MAb and MLR suppressive activity, supports the idea that the result by the current ELISA may reflect the immunosuppressive state of the patient. However, great discrepancy between plasma FK 506 levels following the Sep-Pak method and allo-MLR suppressive activity,[6] especially in the initial phase after organ transplantation, may need further explanation, such as the presence of MAb reactive metabolites with less immunosuppressive activity or the presence of ELISA interfering substances that cannot be removed completely by the Sep-Pak method.

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Measurement of blood concentrations of FK506 (tacrolimus) and its metabolites in seven liver graft patients after the first dose by h.p.l.c.-MS and microparticle enzyme immunoassay (MEIA)

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1 Blood and urine concentrations of the macrolide immunosuppressant FK506 and its metabolites were measured in seven orthotopic liver transplant patients after the first oral dose of FK506 (0.04 ± 0.02 mg kg^{-1}) used as primary immunosuppressant. A specific h.p.l.c.-MS assay was used, allowing the measurement of parent drug and eight metabolites. Results were compared with those obtained using a microparticle enzyme immunoassay (MEIA).

2 Blood drug concentrations were described by an open two compartment model with first-order absorption giving the following mean data: t_{max}: 1.9 (h), C_{max}: 17.4 (μg l^{-1}), AUC: 328.1 (μg l^{-1} h), $t_{½,1}$: 0.74 (h). The terminal elimination half-life was estimated at about 26 h using the h.p.l.c.-MS assay.

3 The metabolites found in blood were demethyl-FK506 and demethyl-hydroxy-FK506, while in urine FK506 and eight of its metabolites were detected.

Keywords FK506 pharmacokinetics drug metabolism FK506 metabolites h.p.l.c.-MS therapeutic drug monitoring

Introduction

FK506 (tacrolimus, Fujisawa, Osaka, Japan) is a 23-member macrolide lactone ($C_{44}H_{69}NO_{12}$, molecular weight: 803.7 Dalton) with a hemiketal-masked α,β-diketoamide function, currently under clinical investigation as an immunosuppressant in patients after organ transplantation [1] and in immunological diseases [2, 3].

FK506 is metabolized in the intestine and liver to at least nine metabolites [4], primarily by cytochrome P450 3A [5, 6]. Four FK506 metabolites resulting from demethylation and hydroxylation were identified after incubation with rat liver microsomes [7]. *In vivo* demethyl-, di-demethyl-, di-demethyl-hydroxy-FK506 have been detected in the blood and urine of liver graft patients [8]. The demethylated metabolites have immunosuppressive activity up to 10% of that of the parent compound [4].

Immunological methods are currently used to measure blood and plasma FK506 concentrations [9–11] using a monoclonal antibody which cross-reacts with FK506 metabolites [7, 12, 13]. The development of methods specific for FK506 and its metabolites has been complicated by low (<20 μg l^{-1}) blood drug concentrations in patients [14]. Since h.p.l.c. with u.v. detection is not sensitive enough to assay such concentrations in patients [15], h.p.l.c. has been combined with immunoassays [12] or derivatisation with dansylhydrazine [16] to enhance sensitivity. An h.p.l.c.-mass spectrometric method (h.p.l.c.-MS) has been described [8] which allows the concomitant measurement of FK506 and its metabolites.

Since all reported pharmacokinetic data for FK506 are based on potentially non-specific immunoassays their significance may be limited.

The aim of this study was to measure blood concentrations of FK506 and its metabolites using a specific h.p.l.c.-MS method after the first oral FK506 dose, and to compare the results with those obtained using a microparticle enzyme immunoassay (MEIA) [17], which is currently the standard procedure for monitoring FK506 concentrations in patients.

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reagents for this assay were purchased from Abbott. According to the IMx® Tacrolimus assay manual, the sensitivity of this assay is 5 μg l^{-1} (lowest measurable concentration which can be distinguished from zero with 95% confidence). The interassay CV during the study was 12.9% at 15.4 μg l^{-1} and 15.4% at 25.9 μg l^{-1} (n = 10).

Results

Table 1 summarizes the results of liver and kidney function tests as well as haematocrit and haemoglobin values for all patients.

The mean blood drug concentrations measured on the first day after transplantation are shown in Figure 1. In two patients a second increase of the FK506 concentration was observed after 5 and 8 h, respectively, representing about 70% of the corresponding C_{max} value. Statistical tests on the curve fitting iterations indicated a median quadratic error of 10.3

Table 1 Clinical chemistry and haematological measurements in the patients on the first day after transplantation

	Median (n = 7)	*Range min*	*max*
ASAT (u l^{-1})	249	76	2029
ALAT (u l^{-1})	282	60	365
GGT (u l^{-1})	22	16	93
ALP (u l^{-1})	83	69	271
CHS (ku l^{-1})	3.3	2.4	4.1
GLDH (u l^{-1})	45	32	83
Bilirubin (μmol l^{-1})	102	38	448
Creatinine (μmol l^{-1})	97	70	228
Urea (mmol l^{-1})	12	7.5	24
HCT (%)	35	28	40
HB (g dl^{-1})	12	9.5	14

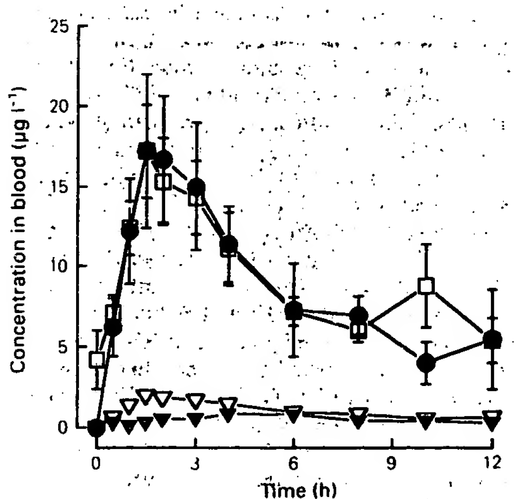

Figure 1 Mean (± s.e. mean) blood FK506 and metabolite concentrations in seven liver graft patients on the first day after transplantation. ● FK506, ▼ 13-demethyl-FK506, ▽ demethyl-hydroxy-FK506 (measured by h.p.l.c.-MS), □ measured by microparticle enzyme immunoassay (MEIA).

(range 0.4–47.4) and a median correlation coefficient of 0.966. The pharmacokinetic parameters obtained from the h.p.l.c.-MS and MEIA measurements are shown in Table 2. Significant differences between the results using the two assays were not detected (Wilcoxon's test). Owing to the limited sampling time values of $t_{1/2,z}$ were determined approximately. The mean value was about 26 h (range 4–60 h) using the h.p.l.c.-MS and 39 h (range 2–58 h) using the MEIA.

Metabolites in blood represented about 20% of the FK506-derived material on the first day after transplantation. The main metabolites found in blood were demethyl-FK506 and demethyl-hydroxy-FK506. After the first FK506 dose no correlations between the biochemical, haematological and pharmacokinetic parameters were found.

In urine only 20% of the FK506-derived material was present as parent drug, reaching concentrations of 0.5 ± 0.15 μg l^{-1} after 4 h. Of the metabolites measured in urine demethyl-FK506 had the highest concentration (maximum of 0.73 ± 0.27 μg l^{-1} after 8 h). Other metabolites were detected by h.p.l.c.-MS at concentrations between 0.2–0.5 μg l^{-1}.

Discussion

The oral absorption of FK506, in contrast to that of cyclosporine, is less dependent on bile production [22]. Previous studies showed that mean trough and peak blood FK506 concentrations were similar in liver transplant patients before and after T-tube clamping [22]. Thus, FK506 can be given orally immediately after liver transplantation. Major problems in studying the phamacokinetics of immunosuppressants in liver transplant patients are the variability in the functional state of the liver graft in the early phase after transplantation [23] and relatively short blood sampling periods imposed by the dosing intervals. Owing to the limited data collection, covering less than one FK506 half-life for some patients, the terminal elimination half-life had to be calculated by extrapolation of the fitted curve and thus represents an approximate value.

Table 2 Pharmacokinetic parameters (median + range) of FK506 and its metabolites based on whole blood concentrations measured by h.p.l.c.-MS and MEIA on the first day after liver transplantation

	C_{max} (μg l^{-1})	t_{max} (h)	$t_{1/2,z}$ (h)	*AUC(0.12 h)* (μg l^{-1} h)
FK506	16.3 (3.6–30)	1.8 (0.9–3.4)	0.76 (0.17–1.34)	280 (60–702)
FK506 (MEIA)	17.4 (7.9–27.2)	1.8 (0.9–2.9)	0.77 (0.35–1.16)	280 (145–1140)
Demethyl-hydroxy-FK506	1.9 (1.3–3.0)	2.1 (0.9–3.6)	0.92 (0.13–1.29)	26 (13–52)
Demethyl-FK506	0.82 (0.6–1.0)	3.9 (1.3–7.6)	1.27 (0.05–1.27)	8.7 (2.4–22.5)

Methods

Patients and blood sample collection

Seven liver transplant patients (three female and four male) under primary FK506 immunosuppression were included in the study. Their mean age was 43 years (range 20 to 61 years). The patients had a mean weight of 62 kg (range 36 to 80 kg). Patients who had to take other drugs known either to inhibit or induce cytochrome P450 3A were excluded [18]. Exceptions were methylprednisolone and prednisolone as described below.

The first FK506 dose was given 6 h after revascularization of the liver graft. Multiple blood samples were drawn immediately into EDTA containing tubes, before administration of FK506 and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12 h thereafter.

Urine samples were collected from six patients at 4 and 8 h after the first dose. Blood and urine samples were frozen at −20° C until assayed.

Liver function (serum bilirubin, activities of alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), cholinesterase (CHS), glutamate dehydrogenase (GLDH)) and kidney function (serum urea and creatinine), haematocrit (HCT) and haemoglobin (HB) were measured daily using standard methods (Institut für Klinische Chemie, Medizinische Hochschule Hannover, Hannover, Germany).

Dosage

FK506 was administered orally every 12 h. The first FK506 dose was 0.04 ± 0.02 mg kg^{-1} (mean ± s.d.). Doses were adjusted subsequently according to the clinical condition of the patient and blood FK506 concentrations as measured by microparticle enzyme immunoassay (Abbott, Abbott Park, IL). An i.v. bolus of 500 mg methylpredisolone was given to all patients after reperfusion. Five patients received prednisolone (0.27 to 0.67 mg kg^{-1}) and one received azathioprine (1 mg kg^{-1}).

Data analysis

Peak blood drug concentration (C_{max}), time to peak concentration (t_{max}), AUC(0,12 h), initial half-life ($t_{1/2,1}$), and terminal half-life ($t_{1/2,z}$) were determined for FK506, demethyl-FK506 and demethyl-hydroxy-FK506. The concentrations of all other drug metabolites in blood were below the detection limit of the h.p.l.c.-MS assay. A biexponential distribution function with first-order input was fitted to the blood drug concentration data by nonlinear regression using the Topfit program (Version 2.0, Gödecke, Freiburg, Germany). This model was selected according to the Akaike and Schwarz criteria. For data derived using the HPLC-MS assay a weighting factor of w = 1/sqrt y(i) was used and for those derived using the MEIA assay the factor was w = 1. The weighting factors were selected according to the variance characteristics of the assays.

Assays of FK506 and its metabolites

The h.p.l.c.-MS assay was based upon a previously described method [8] which was slightly modified. 32-*O*-acetyl-FK506 was synthesized for use as an internal standard (IS) [8]. To 1 ml of each blood or urine sample 10 µl internal standard solution (0.8 mg l^{-1} in acetonitrile/water pH 3.0, 70/30 v/v) and 2 ml of a methanol/water solution (70/30 v/v) saturated with zinc sulphate were added. Samples were vortexed for 20 s and centrifuged (2000 *g*, 2 min). The supernatants were drawn through glass extraction columns filled with LiChroprep® (C_8, 25–40 µm particle size, Merck, Darmstadt, Germany), which had previously been primed with 3 ml acetonitrile and 3 ml water pH 3.0. The samples were washed with 3 ml methanol/water pH 3.0 (50/50 v/v) and 1 ml hexane. FK506 and its metabolites were eluted by centrifuging 1.5 ml dichloromethane through the columns. The dichloromethane was evaporated at 50° C under a stream of nitrogen and the residues were dissolved in 300 µl acetonitrile/water pH 3.0 (70/30 v/v), washed with 500 µl hexane and injected into the h.p.l.c.-MS system.

The h.p.l.c.-MS system consisted of L-6200 and L-6000 h.p.l.c. pumps and a 655 A40 injector (Merck-Hitachi, Darmstadt), connected through a particle-beam interface to a 5989A mass spectrometer (Hewlett-Packard, Waldbronn). Data were recorded and analyzed using MS-ChemStation Software (Version C.01.05, Hewlett-Packard).

The extract (99 µl) was injected onto a Spherical® C_{18} column (3.9 × 150 mm, 5 µm particle size, Waters Millipore, Milford, USA). FK506 and its metabolites were eluted isocratically with methanol/water (90/10 v/v) at a flow rate of 0.4 ml min^{-1}. The MS parameters were adjusted as described previously [8]. For chemical ionization, methane (purity >99.5%, Messer, Griesheim) was used as reagent gas and negative ions were detected. The MS was run in the selected ion mode (SIM) and focussed on the following masses: 775.7 amu (di-demethyl-FK506), 789.7 amu (demethyl-FK506), 791.7 amu (di-demethyl-hydroxy-FK506), 803.7 amu (FK506), 805.7 amu (demethyl-hydroxy-FK506), 807.7 amu (di-demethyl-di-hydroxy-FK506), 819.7 amu (hydroxy-FK506), 821.7 amu (di-hydroxy-demethyl-FK506), 835.7 amu (di-hydroxy- FK506), 845.7 amu (32-*O*-acetyl-FK506; internal standard).

For quality control during the study two of each precision and calibration control samples were run for every six study samples. The calibration curve in whole blood comprised six data points at concentrations of 0, 0.5, 1, 5, 25, 100 µg l^{-1} with n = 5 for each concentration. The lower limit of quantification was 0.2 µg l^{-1} for FK506 and its metabolites, the calibration curve was linear from 0.2 to 100 µg l^{-1} (r = 0.98), recovery of FK506 from whole blood was 71 ± 24% (mean ± s.d.) at 25 µg l^{-1} and the interassay CV was 11.1% at 5 µg l^{-1} and 13.1% at 25 µg l^{-1} (n = 16).

An aliquot of each blood sample was also assayed by microparticle enzyme immunoassay using the Abbott IMx® analyzer (Abbott, Abbott Park, IL). All

The appearance of secondary peaks in some patients may be explained by differences in absorption kinetics arising from variability in gastric motility and poor dissolution of FK506, as has been reported by cyclosporine [24].

It is difficult to compare our results with other studies of the pharmacokinetics of FK506 [25] since most of these used plasma as the analytical matrix and applied different pharmacokinetic models. All of these previous studies measured FK506 by non-specific immunological assay. Thus, no data on the pharmacokinetics of FK506 metabolites are available. Compared with blood concentrations of cyclosporine metabolites, which reach higher trough values than the parent compound [26], the FK506 metabolite fraction in blood was relatively small. This may be explained either by a limited metabolic capacity of the freshly transplanted liver and/or by inhibition of cytochrome P450 3A4 following the 500 mg i.v. bolus of methylprednisolone prior to FK506 administration [18, 27]. The main 13-demethyl- and demethyl-hydroxy-metabolites of FK506 measured by h.p.l.c.-MS after the first FK506 dose show virtually no crossreactivity with the monoclonal antibody used in immunoassays [7, 13]. This and their low concentrations in blood explains why both assays gave similar results in this study (Figure 1). However, there is evidence that in cholestasis the metabolites contribute significantly to concentrations measured by immunoassay [28, 29].

Although our estimate of the terminal half-life of FK506 is based on sparse data, the finding of values in excess of 10 h agrees with other recent reports [29]. The clinical use of FK506 is associated with the danger of severe side effects such as nephro- or neurotoxicity [30]. There is limited evidence that adverse effects correlate with elevated FK506 blood concentrations [28, 31], especially in patients with impaired liver function [23, 32]. Because of the potential for drug accumulation due to the long half-life of FK506 and the possibility of severe toxicity, therapeutic drug monitoring of FK506 may be advisable, at least in the early post-operative phase after liver transplantation.

Fruitful discussions with Dr A. Möller (Fujisawa GmbH, Germany) are appreciated.

The study was supported by the Deutsche Forschungsgemeinschaft, grant SFB 265, project A7.

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(*Received 25 April 1994, accepted 9 August 1994*)

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A Whole Blood FK 506 Assay for the IMx® Analyzer

F.C. Grenier, J. Luczkiw, M. Bergmann, S. Lunetta, M. Morrison, D. Blonski, K. Shoemaker, and M. Kobayashi

FK 506 is a novel immunosuppressant discovered in 1984 by the Fujisawa Pharmaceutical Co, Ltd.[1] It is useful for the treatment of rejection following transplantation, graft vs host disease, and autoimmune diseases.[2] Cyclosporine (CyA) has also been used successfully to control rejection and during CyA therapy monitoring the whole blood concentration of CyA is an important aspect of clinical care.[3] Like CyA FK 506 is predominately sequestered in blood cells and it is expected that monitoring blood concentrations of FK 506 will be equally important for patients receiving this drug.

Fujisawa has described an enzyme-linked immunosorbent assay (ELISA) methodology to measure FK 506 comprised of (1) an ELISA plate coated with anti-FK 506 antibodies, (2) an FK 506-horseradish peroxidase conjugate that competes with free FK 506, and (3) an appropriate substrate for the peroxidase.[4] While the ELISA is effective, a semiautomated immunologic technology would be more precise and have greater throughput.

The Abbott IMx analyzer is one such technology and the MEIA format is a proprietary assay configuration that runs on the IMx. A semiautomated whole blood assay for FK 506 described here provides a precise and reliable means of measuring blood FK 506 concentrations in patient samples.

ASSAY DESCRIPTION

The FK 506 MEIA for the IMx analyzer utilizes four reagents: (1) a precipitation reagent to extract FK 506 from whole blood, (2) a capture reagent consisting of latex microparticles to which FK 506 antibodies (Fujisawa clone 1-60-46) have been covalently attached, (3) an FK 506-alkaline phosphatase conjugate reagent, and (4) an enzyme substrate reagent consisting of 4-methylumbelliferyl phosphate. The typical assay sequence is as follows.

FK 506 standards or whole blood patient samples are mixed vigorously with the precipitation reagent and the precipitates formed are removed by centrifugation. The clear organic supernatant is decanted directly into an IMx sample cartridge that has been preloaded into an IMx carousel.

The automated assay sequence on the IMx analyzer is then initiated by pushing the RUN button on the IMx analyzer. In the first step, the extracted sample is mixed with the microparticle reagent. This is followed by an incubation period during which the FK 506 in the sample binds to the antibody binding sites on the microparticles. An aliquot of the above mixture is then transferred to a glass fiber filter on which the microparticles adhere. Following a wash step, the FK 506-alkaline phosphatase conjugate is added to the filter. Conjugate that does not bind to the remaining antibody binding sites on the microparticles is then removed by a second wash step. The substrate reagent is added to the filter and the rate of appearance of fluorescent product is measured by front surface fluorescence measurements and is inversely proportional to the amount of the analyte present in the patient sample or standard.

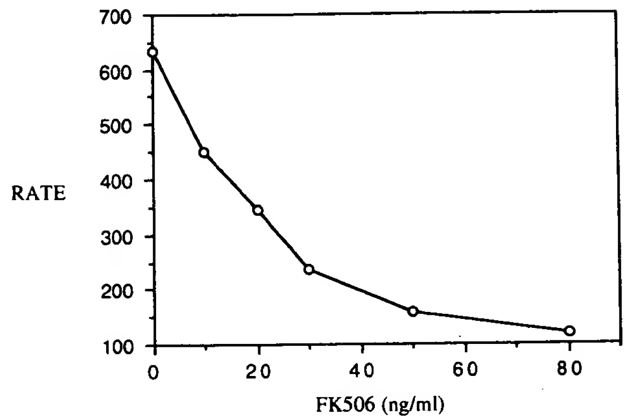

Fig 1. FK 506 MEIA calibration curve.

ASSAY PERFORMANCE

A typical calibration curve is shown in Fig 1 and is stable for at least 2 weeks in the IMx. Following the manual extraction step, approximately 30 minutes are required to analyze 23 samples. Typical assay performance was as follows.

Precision analysis gave coefficients of variance (CVs) of 11.8, 9.6, and 8.1% at FK 506 concentrations of 15, 25, and 65 ng/mL, respectively. The average recovery from 5 to 80 ng/mL was 95% and the sensitivity was 3.3 ng/mL at a 95% confidence level. Lipid interference was under 11% at 1,000 mg/dL triglycerides and bilirubin interference was under 6% at 40 mg/dL bilirubin. Interference caused by whole blood protein concentrations ranging from 11 to 26 g/dL was under 10%. Analysis of whole blood FK 506 samples from liver transplant patients gave concentrations ranging from less than 5 ng/mL to greater than 100 ng/mL.

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Seventy-five percent of the samples were below 30 ng/mL, however, with an average value of 21 ng/mL.

CONCLUSIONS

The whole blood FK 506 assay described above offers convenience, reliability, and good sample throughput. Studies in the clinical setting will ultimately define the best assay specifications. Differences between transplant types or dosage regimens, for example, could necessitate changes in the dynamic range of the assay. Equally important, but poorly understood, will be the importance of FK 506 metabolism. The relative immunosuppressive potencies of FK 506 metabolites and their corresponding cross-reactivities in this assay could also necessitate additional assay development.

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